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First Inventor or Application Identifier

Stephen C. Goshorn

Title

STREPTAVIDIN EXPRESSED GENE FUSIONS AND  
METHODS OF USE THEREOF

Express Mail Label No.

EL615228586US

S-1 U.S. PTO  
5/5/987**APPLICATION ELEMENTS**

See MPEP chapter 600 concerning utility patent application contents.

**ADDRESS TO:**Box Patent Application  
Assistant Commissioner for Patents  
Washington, D.C. 202311.  General Authorization Form & Fee Transmittal  
(Submit an original and a duplicate for fee processing)6.  Microfiche Computer Program (Appendix)2.  Specification [Total Pages] **58**  
(preferred arrangement set forth below)  
- Descriptive Title of the Invention  
- Cross References to Related Applications  
- Statement Regarding Fed sponsored R & D  
- Reference to Microfiche Appendix  
- Background of the Invention7. Nucleotide and Amino Acid Sequence Submission  
(if applicable, all necessary)

- a.  Computer-Readable Copy
- b.  Paper Copy (identical to computer copy)
- c.  Statement verifying identity of above copies

- Brief Summary of the Invention
- Brief Description of the Drawings (if filed)
- Detailed Description
- Claim(s)
- Abstract of the Disclosure

3.  Drawing(s) (35 USC 113) [Total Sheets] **22****ACCOMPANYING APPLICATION PARTS**4. Oath or Declaration [Total Pages] **22**8.  Assignment Papers (cover sheet & document(s))

- a.  Newly executed (original or copy)
- b.  Copy from a prior application (37 CFR 1.63(d))  
(for continuation/divisional with Box 17 completed)  
i.  DELETION OF INVENTOR(S)  
Signed statement attached deleting  
inventor(s) named in the prior application,  
see 37 CFR 1.63(d)(2) and 1.33(b)

9.  37 CFR 3.73(b) Statement  
(when there is an assignee)  Power of Attorney5. Incorporation By Reference (useable if box 4b is checked) The entire disclosure of the prior application,  
from which a copy of the oath or declaration is supplied  
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the accompanying application and is hereby incorporated  
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17. If a CONTINUING APPLICATION, check appropriate box and supply the requisite information below and in a preliminary amendment

Continuation  Divisional  Continuation-In-Part (CIP) of prior Application No. \_\_\_\_\_

Prior application information: Examiner \_\_\_\_\_ Group / Art Unit \_\_\_\_\_

Claims the benefit of Provisional Application No. **60/137,900, filed June 7, 1999 and 60/168,976,**

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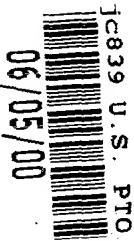
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Enclosures:

Postcard  
Form PTO/SB/05  
Specification, Claims, Abstract (58 pages)  
22 Sheets of Drawings (1-19)  
Sequence Listing  
Declaration Regarding Sequence Listing  
Computer Readable Diskette



STREPTAVIDIN EXPRESSED GENE FUSIONS AND  
METHODS OF USE THEREOF

TECHNICAL FIELD

The present invention relates generally to streptavidin expressed gene fusion constructs, and more particularly, to genomic streptavidin expressed gene fusions and methods of using these constructs in diagnostic and therapeutic applications.

BACKGROUND OF THE INVENTION

Streptavidin ("SA") is a 159 amino acid protein produced by 10 *Streptomyces avidinii*, and which specifically binds water-soluble biotin (Chaiet *et al.*, *Arch. Biochem. Biophys.* 106:1-5, 1964). Streptavidin is a nearly neutral 64,000 dalton tetrameric protein. Accordingly, it consists of four identical subunits each having an approximate molecular mass of 16,000 daltons (Sano and Cantor, *Proc. Natl. Acad. Sci. USA* 87:142-146, 1990). Streptavidin shares some common characteristics with avidin, 15 such as molecular weight, subunit composition, and capacity to bind biotin with high affinity ( $K_D \approx 10^{-15}$ ) (Green, *Adv. Prot. Chem.* 29:85-133, 1975). Further, while streptavidin and avidin differ in their amino acid compositions, both have an unusually high content of threonine and tryptophan. In addition, streptavidin differs from avidin in that it is much more specific for biotin at physiological pH, likely due to the absence 20 of carbohydrates on streptavidin. Various comparative properties and isolation of avidin and streptavidin are described by Green *et al.*, *Methods in Enzymology* 184:51-67, 1990 and Bayer *et al.*, *Methods in Enzymology* 184:80-89, 1990.

The streptavidin gene has been cloned and expressed in *E. coli* (Sano and Cantor, *Proc. Natl. Acad. Sci. USA* 87(1):142-146, 1990; Agarana, *et al.*, *Nucleic Acids Res.* 14(4):1871-1882, 1986). Fusion constructs of streptavidin, and truncated forms thereof, with various proteins, including single-chain antibodies, have also been expressed in *E. coli* (Sano and Cantor, *Biotechnology (NY)* 9(12):1378-1381, 1991; Sano and Cantor, *Biochem. Biophys. Res. Commun.* 176(2):571-577, 1991; Sano, *et al.*, *Proc. Natl. Acad. Sci. USA* 89(5):1534-1538, 1992; Walsh and Swaisgood, *Biotech.*

*Bioeng.* 44:1348-1354, 1994; Le, *et al.*, *Enzyme Microb. Technol.* 16(6):496-500, 1994; Dubel, *et al.*, *J. Immunol. Methods* 178(2):201-209, 1995; Kipriyanov, *et al.*, *Hum. Antibodies Hybridomas* 6(3):93-101, 1995; Kipriyanov, *et al.*, *Protein Eng.* 9(2):203-211, 1996; Ohno, *et al.*, *Biochem. Mol. Med.* 58(2):227-233, 1996; Ohno and Meruelo, 5 *DNA Cell Biol.* 15(5):401-406, 1996; Pearce, *et al.* *Biochem. Mol. Biol. Int.* 42(6):1179-1188, 1997; Koo, *et al.*, *Applied Environ. Microbiol.* 64(7):2497-2502, 1998) and in other organisms (Karp, *et al.*, *Biotechniques* 20(3):452-459, 1996). Sano and Cantor (PNAS, *supra*) found that expression of full-length forms of streptavidin was lethal to *E. coli* host cells and, when capable of being expressed in truncated forms (e.g., under a 10 T7 promoter system), only poor and varied expression was observed and the protein remained in inclusion bodies. However, there are also published reports of the expression of soluble streptavidin in *E. coli* (Gallizia *et al.*, *Protein Expr. Purif.* 14(2):192-196, 1998; Veiko *et al.*, *Bioorg. Khim.* 25(3):184-188, 1999). Those of skill in the art have frequently used "core streptavidin" (residues 14-136), or similar 15 truncated forms, in the preparation of fusion constructs. The basis of the use of core residues 14-136 has been the observation that streptavidin preparations purified from the culture medium of *S. avidinii* have usually undergone proteolysis at both the N- and C-termini to produce this core structure, or functional forms thereof (Argarana *et al.*, *supra*).

20 Presently, preparations of streptavidin expressed gene fusions are usually made by expressing a core streptavidin-containing construct in bacteria, wherein inclusion bodies are formed. Such production has several disadvantages, including the rigor and expense of purifying from inclusion bodies, the necessity of using harsh denaturing agents such as guanidine hydrochloride, and the difficulty in 25 scaling up in an economical fashion. To a lesser extent, there has also been reported periplasmic expression of core streptavidin-containing constructs in soluble form (Dubel, *et al.*, *supra*).

30 Therefore, there exists a need in the art for easy, cost effective, and scaleable methods for the production of streptavidin fusion proteins. Accordingly, the present invention provides several key advantages. For example, in one embodiment, a genomic streptavidin expressed gene fusion is expressed as a soluble protein into the

periplasmic space of bacteria and undergoes spontaneous folding. Accordingly, such expression offers the advantage that the periplasm is a low biotin environment and one need not purify and refold the protein under harsh denaturing conditions that may prove fatal to the polypeptide encoded by a heterologous nucleic acid molecule fused to the 5 genomic streptavidin nucleic acid molecule. The present invention fulfills this need, while further providing other related advantages.

## SUMMARY OF THE INVENTION

The present invention generally provides expression cassettes and fusion constructs encoded thereby comprising genomic streptavidin. In one aspect the present 10 invention provides a vector construct for the expression of streptavidin fusion proteins, comprising a first nucleic acid sequence encoding at least 129 amino acids of streptavidin (Figure 4), or a functional variant thereof, a promoter operatively linked to the first nucleic acid sequence, and a cloning site for, or with, insertion of a second nucleic acid sequence encoding a polypeptide to be fused with streptavidin, interposed 15 between the promoter and the first nucleic acid sequence. Alternatively, the second nucleic acid may encode the streptavidin portion of the construct and the first nucleic acid encodes a polypeptide to be fused with streptavidin.

In certain embodiments, the promoter is inducible or constitutive. In other embodiments, the first nucleic acid sequence encodes at least amino acids 14 to 20 150, 14 to 151, 14 to 152, 14 to 153, 14 to 154, 14 to 155, 14 to 156, 14 to 157, or 14 to 158 of streptavidin, Figure 4. In yet other embodiments, the first nucleic acid sequence encodes at least amino acids 5 to 150-158 of Figure 4 or 1 to 150-158 of Figure 4.

Host cells containing genomic streptavidin expression cassettes are also provided as are fusion proteins expressed by the same. In certain embodiments fusion 25 proteins comprising single chain antibodies are provided. In yet other embodiments the single chain antibodies are directed to a cell surface antigen. In yet other embodiments the single chain antibodies are directed to cell surface antigens, or cell-associated stromal or matrix antigens, including, but not limited to, CD20, CD22, CD45, CD52, CD56, CD57, EGP40 (or EPCAM or KSA), NCAM, CEA, TAG-72, mucins (MUC-1 30 through MUC-7), -HCG, EGF receptor, IL-2 receptor, her2/neu, Lewis Y, GD2, GM2,

tenascin, sialylated tenascin, somatostatin, activated tumor stromal antigen, or neoangiogenic antigens.

In other aspects of the present invention, methods for targeting a tumor cell are provided, comprising the administration of a fusion protein, said fusion protein 5 comprising at least a first and a second polypeptide joined end to end, wherein said first polypeptide comprises at least 129 amino acids of streptavidin (Figure 4), or conservatively substituted variants thereof, wherein said second polypeptide is a polypeptide which binds a cell surface protein on a tumor cell, wherein the fusion protein binds the cell surface protein on a tumor cell and wherein the streptavidin 10 portion of the fusion protein is capable of binding biotin. In certain embodiments, the second polypeptide is an antibody or antigen-binding fragment thereof.

In other aspects of the present invention, pharmaceutical compositions, comprising genomic streptavidin fusion constructs are provided.

These and other aspects of the present invention will become evident 15 upon reference to the following detailed description and attached drawings.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of a heterologous protein-genomic streptavidin expressed gene construct.

Figure 2 is a schematic representation of a single chain antibody- 20 genomic streptavidin fusion construct.

Figure 3 is a schematic representation of the pEX94B expression vector containing a single chain antibody(huNR-LU-10)-genomic streptavidin fusion construct.

Figure 4 is the sequence of genomic streptavidin (SEQ ID NO: 1) 25 including the signal sequence and predicted amino acid sequence (SEQ ID NO: 2).

Figure 5 is a schematic representation of the construction of the pKKlac/pelB vector.

Figure 6 is a schematic representation of the construction of the pEX-1 vector.

Figure 7 is a schematic representation of the construction of the pEX-SA318 and pEX-scFv3.2.1 vectors.

Figure 8 is a schematic representation of the construction of the pEX94B vector.

5 Figure 9 is a schematic representation of the construction of the pEX94B neo vector.

Figure 10 represents the determined nucleic acid sequence (SEQ ID NO: 3) and predicted amino acid sequence (SEQ ID NO: 4) for the huNR-LU-10 single chain antibody-genomic streptavidin fusion. The streptavidin regulatory region, signal 10 sequence, and coding sequence are noted as are the various linkers and light and heavy chains of the single chain antibody.

Figures 11A and 11B are the determined nucleic acid (SEQ ID NO: 5) and predicted amino acid sequences (SEQ ID NO: 6) of a B9E9 scFvSA fusion construct, with the pKOD linker between  $V_L$  and  $V_H$ . Linkers are boxed and the 15 orientation is  $V_L$ -linker- $V_H$ -linker-Streptavidin.

Figure 11C is an expression cassette comprising the nucleic acid sequences (SEQ ID NO: 7) and predicted amino acid sequences (SEQ ID NO: 8) of a B9E9 scFvSA fusion construct encoding  $V_H$ -linker- $V_L$ -linker-Streptavidin.

Figure 12 is a scanned image representing SDS-PAGE analysis of 20 huNR-LU-10 scFvSA.

Figure 13 is graphic representation of size exclusion HPLC analysis of huNR-LU-10 scFvSA.

Figure 14 is a plot illustrating a competitive immunoreactivity assay of huNR-LU-10 scFvSA (97-20.0 and 98-01.0) as compared to huNR-LU-10 mAb.

25 Figure 15 is a plot illustrating the rate of dissociation of DOTA-biotin from huNR-LU-10 scFvSA (97-13.0) as compared to recombinant streptavidin (r-SA).

Figure 16 is a graph illustrating biodistribution of pretargeted huNR-LU-10 scFvSA.

Figure 17 is a graph depicting blood clearance and tumor uptake of 30 huNR-LU-10 scFvSA versus a chemically conjugated form (mAb/SA).

Figure 18 is a bar graph illustrating biodistribution of pretargeted B9E9 scFvSA.

Figure 19 is a scanned image of SDS-PAGE analysis of scFvSA fusion protein expression in the presence and absence of FkpA.

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#### DETAILED DESCRIPTION OF THE INVENTION

Prior to setting forth the invention, it may be helpful to an understanding thereof to set forth definitions of certain terms that will be used hereinafter.

"Core streptavidin," as used herein, refers to a streptavidin molecule 10 consisting of the central amino acid residues 14-136 of streptavidin of Figure 4 and also of Figure 3 of U.S. Patent No. 4,839,293 and deposited at ATCC number X03591.

"Genomic streptavidin," as used herein, refers to a sequence comprising 15 at least 129 residues of the sequence set forth in Figure 4. Accordingly, genomic streptavidin refers to streptavidin molecules that have N-terminal, C-terminal, or both N- and C-terminal extensions of core streptavidin. The N- and C-terminal extensions may comprise any number of amino acids selected from 1 to 13, 137 to 159, and in some cases -1 to -24 of Figure 4.

The genomic streptavidin molecules of the subject invention also include 20 variants (including alleles) of the native protein sequence. Briefly, such variants may result from natural polymorphisms or may be synthesized by recombinant DNA methodology, and differ from wild-type protein by one or more amino acid substitutions, insertions, deletions, or the like. Variants generally have at least 75% nucleotide identity to native sequence, preferably at least 80%-85%, and most preferably at least 90% nucleotide identity. Typically, when engineered, amino acid 25 substitutions will be conservative, *i.e.*, substitution of amino acids within groups of polar, non-polar, aromatic, charged, etc. amino acids. With respect to homology to the native sequence, variants should preferably have at least 90% amino acid sequence identity, and within certain embodiments, greater than 92%, 95%, or 97% identity. Such amino acid sequence identity may be determined by standard methodologies, 30 including use of the National Center for Biotechnology Information BLAST search

methodology available at [www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov), using default parameters. The identity methodologies most preferred are those described in U.S. Patent 5,691,179 and Altschul *et al.*, *Nucleic Acids Res.* 25:3389-3402, 1997.

As will be appreciated by those skilled in the art, a nucleotide sequence and the encoded genomic streptavidin or variant thereof may differ from known native sequence, due to codon degeneracies, nucleotide polymorphisms, or amino acid differences. In certain embodiments, variants will preferably hybridize to the native nucleotide sequence at conditions of normal stringency, which is approximately 25-30°C below T<sub>m</sub> of the native duplex (e.g., 5X SSPE, 0.5% SDS, 5X Denhardt's solution, 50% formamide, at 42°C or equivalent conditions; *see generally*, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, 2nd ed., Cold Spring Harbor Press, 1989; Ausubel *et al.*, *Current Protocols in Molecular Biology*, Greene Publishing, 1995). By way of comparison, low stringency hybridizations utilize conditions approximately 40°C below T<sub>m</sub>, and high stringency hybridizations utilize conditions approximately 10°C below T<sub>m</sub>.

A "polypeptide," as used herein, refers to a series of amino acids of five or more.

An "isolated nucleic acid molecule" refers to a polynucleotide molecule in the form of a separate fragment or as a component of a larger nucleic acid construct, that has been separated from its source cell (including the chromosome it normally resides in) at least once, and preferably in a substantially pure form. Nucleic acid molecules may be comprised of a wide variety of nucleotides, including DNA, RNA, nucleotide analogues, or combination thereof.

The term "heterologous nucleic acid sequence", as used herein, refers to at least one structural gene operably associated with a regulatory sequence such as a promoter. The nucleic acid sequence originates in a foreign species, or, in the same species if substantially modified from its original form. For example, the term "heterologous nucleic acid sequence" includes a nucleic acid originating in the same species, where such sequence is operably associated with a promoter that differs from the natural or wild-type promoter.

An "antibody," as used herein, includes both polyclonal and monoclonal antibodies; primatized (e.g., humanized); murine; mouse-human; mouse-primate; and chimeric; and may be an intact molecule, a fragment thereof (such as scFv, Fv, Fd, Fab, Fab' and F(ab)'<sub>2</sub> fragments), or multimers or aggregates of intact molecules and/or fragments; and may occur in nature or be produced, e.g., by immunization, synthesis or genetic engineering; an "antibody fragment," as used herein, refers to fragments, derived from or related to an antibody, which bind antigen and which in some embodiments may be derivatized to exhibit structural features that facilitate clearance and uptake, e.g., by the incorporation of galactose residues. This includes, e.g., F(ab), F(ab)'<sub>2</sub>, scFv, light chain variable region (V<sub>L</sub>), heavy chain variable region (V<sub>H</sub>), and combinations thereof.

The term "protein," as used herein, includes proteins, polypeptides and peptides; and may be an intact molecule, a fragment thereof, or multimers or aggregates of intact molecules and/or fragments; and may occur in nature or be produced, e.g., by synthesis (including chemical and/or enzymatic) or genetic engineering.

## **A. Streptavidin Genes and Gene Products**

### *1. Streptavidin Nucleic Acid Molecules and Variants Thereof*

The present invention provides streptavidin fusion constructs that include streptavidin nucleic acid molecules of various lengths, which, in certain embodiments, are constructed from full-length genomic streptavidin nucleic acid molecules available in the art and specifically described in U.S. Patent Nos. 4,839,293; 5,272,254, and ATCC Accession number X03591.

Variants of streptavidin nucleic acid molecules, provided herein, may be engineered from natural variants (e.g., polymorphisms, splice variants, mutants), synthesized or constructed. Many methods have been developed for generating mutants (see, generally, Sambrook *et al.*, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Press, 1989, and Ausubel, *et al. Current Protocols in Molecular Biology*, Greene Publishing Associates and Wiley-Interscience, New York, 1995). Briefly, preferred methods for generating nucleotide substitutions utilize an

oligonucleotide that spans the base or bases to be mutated and contains the mutated base or bases. The oligonucleotide is hybridized to complementary single stranded nucleic acid and second strand synthesis is primed from the oligonucleotide. The double-stranded nucleic acid is prepared for transformation into host cells, typically *E. coli*, but alternatively, other prokaryotes, yeast or other eukaryotes. Standard methods of screening and isolation and sequencing of DNA were used to identify mutant sequences.

Similarly, deletions and/or insertions of the streptavidin nucleic acid molecule may be constructed by any of a variety of known methods as discussed, *supra*. For example, the nucleic acid molecule can be digested with restriction enzymes and religated, thereby deleting or religating a sequence with additional sequences (e.g., linkers), such that an insertion or large substitution is made. Other means of generating variant sequences may be employed using methods known in the art, for example those described in Sambrook *et al.*, *supra*; Ausubel *et al.*, *supra*. Verification of variant sequences is typically accomplished by restriction enzyme mapping, sequence analysis, or probe hybridization. In certain aspects, variants of streptavidin nucleic acid molecules whose encoded product is capable of binding biotin, are useful in the context of the subject invention. In other aspects, the ability of the variant streptavidin to bind biotin may be increased, decreased or substantially similar to that of native streptavidin. In yet other embodiments, the ability to bind biotin is not required, provided that the variant form retains the ability to self-assemble into a typical tetrameric structure similar to that of native streptavidin. Such tetrameric structures have a variety of uses such as the formation of tetravalent antibodies when fused to sequences encoding an antibody or fragment thereof.

25        2.        *Genomic Streptavidin and Expression Cassettes Containing the Same*

A genomic streptavidin fusion construct expression cassette of the present invention may be generated by utilizing the full gene sequence of the streptavidin gene, or variant thereof. In certain embodiments, the expression cassette contains a nucleic acid sequence encoding at least 129 contiguous amino acids of Figure 4 or functional variants thereof. In various other embodiments, the nucleic acid

sequence encodes at least amino acid residues 14 to 140 of Figure 4. In a further embodiment, the nucleic acid sequence encodes at least amino acids 14 to 150, 14 to 151, 14 to 152, 14 to 153, 14 to 154, 14 to 155, 14 to 156, 14 to 157, 14 to 158, or 14 to 159 of streptavidin, Figure 4. In yet other embodiments, the nucleic acid sequence 5 encodes at least amino acids 10 to 150-158 of Figure 4, or 5 to 150-158 of Figure 4 or 1 to 150-158 of Figure 4. In yet other embodiments, the nucleic acid sequence encodes at least amino acid residues 1 to 159 of Figure 4. In still yet other embodiments, the expression cassette comprises a nucleic acid sequence that encodes at least 10 amino acids of residues -1 to -24 of Figure 4.

10 As noted above, the genomic streptavidin encoding nucleic acid molecules of the subject invention may be constructed from available streptavidin sequences by a variety of methods known in the art. A preferred method is amplification (*e.g.*, polymerase chain reaction (PCR)) to selectively amplify the individual regions and place these in cloning vectors such as pCR2.1 (Invitrogen).  
15 Moreover, such PCR reactions can be performed in a variety of ways such that the primers used for amplification contain specific restriction endonuclease sites to facilitate insertion into a vector.

Further, a variety of other methodologies besides PCR may be used to attain the desired construct. For example, one skilled in the art may employ isothermal 20 methods to amplify the nucleotide sequence of interest, using existing restriction endonuclease sites present in the nucleotide sequence to excise and insert sequences, or by the introduction of distinct restriction endonuclease sites by site-directed mutagenesis followed by excision and insertion. These and other methods are described in Sambrook *et al.*, *supra*; Ausubel, *et al.*, *supra*. Briefly, one methodology 25 is to generate single-stranded streptavidin encoding DNA, followed by annealing a primer, which is complementary except for the desired alteration (*e.g.*, a small insertion, deletion, or mutation such that a unique restriction site is created between the domains). Bacterial cells are transformed and screened for those cells which contain the desired construct. This construct is then digested to liberate the desired sequences, 30 which can then be purified and religated into the appropriate orientation.

One of skill in the art would recognize that the absolute length of the genomic streptavidin is only a secondary consideration when designing an expression cassette, as compared to utilizing a form which is capable of binding biotin and capable of expressing into the periplasmic space of a bacterial host. Such constructs can be 5 readily tested for their ability to bind biotin and maintain solubility in the periplasmic space by assays known in the art and those described herein. Accordingly, experiments such as, measuring biotin binding capacity and biotin dissociation rate are well known in the art and applicable in this regard. Briefly, such constructs can be tested for their ability to bind biotin by a variety of means, including labeling the fusion protein with a 10 subsaturating level of radiolabeled biotin, then adding a 100-fold saturating level of biocytin to initiate dissociation. The free radiolabeled biotin is measured at timed intervals.

#### **B. Vectors, Host Cells and Methods of Expressing and Producing Protein**

The expression cassette of the present invention need not necessarily 15 contain a promoter, but upon insertion into a vector system the sequence contained within the cassette must be capable of being expressed once associated with a promoter or other regulatory sequences. In one embodiment, the expression cassette itself comprises a promoter. Further, the cassette preferably contains a cloning site for the insertion of a heterologous nucleic acid sequence to be fused/linked to the genomic 20 streptavidin encoding sequence. One exemplary cassette is set forth in Figure 1. However, it should be noted that the cloning site need not be 5' of the genomic streptavidin sequence, but could be placed 3' of the streptavidin sequence. Thus, an encoded fusion protein could contain the genomic streptavidin polypeptide either N- or C-terminal to the encoded polypeptide fused thereto. Further, while it should be noted 25 that a variety of other nucleic acid sequences can be linked to the genomic streptavidin encoding sequence, in one embodiment the sequence encodes an antibody fragment, and in certain embodiments a single chain antibody (scFv).

In addition to a cloning site, the cassette may include a linker molecule. Linker molecules are typically utilized within the context of fusion proteins and are 30 well known in the art. As exemplified in Figure 2, linkers are typically utilized to

separate the genomic streptavidin sequence from the other sequences linked thereto and to separate the  $V_H$  and the  $V_L$  of the scFv. The linking sequence can encode a short peptide or can encode a longer polypeptide. Preferable linker sequences encode at least two amino acids, but may encode as many amino acids as desired as long as functional activity is retained. In the various embodiments, the linker sequence encodes 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, and 35 amino acids. In certain embodiments an encoded linker may be a standard linker such as  $(\text{Gly}_4\text{Ser})_x$  where x may be any integer, but is preferably 1 to 10. The length and composition can be empirically determined to give the optimum expression and biochemical characteristics. For example, the composition of the linker can be changed to raise or lower the isoelectric point of the molecule. Additionally, one of ordinary skill in the art will appreciate that the length of linker between variable light and heavy chains need be at least long enough to facilitate association between the two domains, while the linker between streptavidin and the antibody fragment may vary from zero amino acids to 100 or more as long as functionality is maintained. Accordingly, the linker between the light and heavy chain is typically greater than five amino acids, and preferably greater than ten, and more preferably greater than fifteen amino acids in length.

The expression cassette may be used in a vector to direct expression in a variety of host organisms. In certain embodiments, the genomic streptavidin expressed gene fusion is produced in bacteria, such as *E. coli*, or mammalian cells (e.g., CHO and COS-7), for which many expression vectors have been developed and are available. Other suitable host organisms include other bacterial species, and eukaryotes, such as yeast (e.g., *Saccharomyces cerevisiae*), plants, and insect cells (e.g., Sf9).

In one embodiment, a DNA sequence encoding a genomic streptavidin fusion protein is introduced into an expression vector appropriate for the host cell. As discussed above, the sequence may contain alternative codons for each amino acid with multiple codons. The alternative codons can be chosen as "optimal" for the host species. Restriction sites are typically incorporated into the primer sequences and are chosen with regard to the cloning site of the vector. If necessary, translational initiation and termination codons can be engineered into the primer sequences.

At a minimum, the vector will contain a promoter sequence. As used herein, a "promoter" refers to a nucleotide sequence that contains elements that direct the transcription of a linked gene. At a minimum, a promoter contains an RNA polymerase binding site. More typically, in eukaryotes, promoter sequences contain 5 binding sites for other transcriptional factors that control the rate and timing of gene expression. Such sites include TATA box, CAAT box, POU box, AP1 binding site, and the like. Promoter regions may also contain enhancer elements. When a promoter is linked to a gene so as to enable transcription of the gene, it is "operatively linked."

The expression vectors used herein include a promoter designed for 10 expression of the proteins in a host cell (e.g., bacterial). Suitable promoters are widely available and are well known in the art. Inducible or constitutive promoters are preferred. Such promoters for expression in bacteria include promoters from the T7 phage and other phages, such as T3, T5, and SP6, and the *trp*, *lpp*, and *lac* operons. Hybrid promoters (see, U.S. Patent No. 4,551,433), such as *tac* and *trc*, may also be 15 used. Promoters for expression in eukaryotic cells include the P10 or polyhedron gene promoter of baculovirus/insect cell expression systems (see, e.g., U.S. Patent Nos. 5,243,041, 5,242,687, 5,266,317, 4,745,051, and 5,169,784), MMTV LTR, CMV IE promoter, RSV LTR, SV40, metallothionein promoter (see, e.g., U.S. Patent No. 4,870,009), ecdysone response element system, tetracycline-reversible silencing 20 system (tet-on, tet-off), and the like.

The promoter controlling transcription of the genomic streptavidin fusion construct may itself be controlled by a repressor. In some systems, the promoter can be derepressed by altering the physiological conditions of the cell, for example, by the addition of a molecule that competitively binds the repressor, or by altering the 25 temperature of the growth media. Preferred repressor proteins include, the *E. coli* lacI repressor responsive to IPTG induction, the temperature sensitive  $\lambda$ cI857 repressor, and the like.

Other regulatory sequences may be included. Such sequences include a transcription termination sequence, secretion signal sequence (e.g., nucleotides 480-551 30 of Figure 2B of U.S. Patent No. 5,272,254), ribosome binding sites, origin of replication, selectable marker, and the like. The regulatory sequences are operationally

associated with one another to allow transcription, translation, or to facilitate secretion. The regulatory sequences of the present invention also include the upstream region of the streptavidin gene as described in U.S. Patent No. 5,272,254 (e.g., nucleic acid residues 174-551 depicted in Figures 2A-2B of U.S. Pat. No. 5,272,254). Accordingly,

5 an upstream sequence of 100 to 300 base pairs may be utilized in expression constructs to facilitate secretion and/or expression. Such an upstream untranslated region is depicted in U.S. Patent No. 5,272,254 Figures 2A and 2B as nucleotides 174-479. In preferred embodiments nucleic acid residues 408-479 of those described above are utilized in the expression construct.

10 In other optional embodiments, the vector also includes a transcription termination sequence. A "transcription terminator region" has either a sequence that provides a signal that terminates transcription by the polymerase that recognizes the selected promoter and/or a signal sequence for polyadenylation.

In one aspect, the vector is capable of replication in the host cells. Thus,

15 when the host cell is a bacterium, the vector preferably contains a bacterial origin of replication. Bacterial origins of replication include the f1-ori and col E1 origins of replication, especially the ori derived from pUC plasmids. In yeast, ARS or CEN sequences can be used to assure replication. A well-used system in mammalian cells is SV40 ori.

20 The plasmids also preferably include at least one selectable marker that is functional in the host. A selectable marker gene includes any gene that confers a phenotype on the host that allows transformed cells to be identified and selectively grown. Suitable selectable marker genes for bacterial hosts include the ampicillin resistance gene (Amp<sup>r</sup>), tetracycline resistance gene (Tc<sup>r</sup>) and the kanamycin resistance gene (Kan<sup>r</sup>). The ampicillin resistance and kanamycin resistance genes are presently preferred. Suitable markers for eukaryotes usually require a complementary deficiency in the host (e.g., thymidine kinase (tk) in tk- hosts). However, drug markers are also available (e.g., G418 resistance and hygromycin resistance).

30 The nucleotide sequence encoding the genomic streptavidin fusion protein may also include a secretion signal (e.g., a portion of the leader sequence, the leader sequence being the upstream region of a gene including a portion of a secretion

signal), whereby the resulting peptide is a precursor protein processed and secreted. The resulting processed protein may be recovered from the periplasmic space or the fermentation medium. Secretion signals suitable for use are widely available and are well known in the art (von Heijne, *J. Mol. Biol.* 184:99-105, 1985; von Heijne, *Eur. J. Biochem.* 133:17-21, 1983). Prokaryotic and eukaryotic secretion signals that are functional in *E. coli* (or other host) may be employed. The presently preferred secretion signals include, but are not limited to, those encoded by the following bacterial genes: streptavidin, pelB (Lei *et al.*, *J. Bacteriol.* 169:4379, 1987), phoA, ompA, ompT, ompF, ompC, beta-lactamase, and alkaline phosphatase.

Other components which increase expression may also be included either within the vector directing expression of the streptavidin fusion or on a separate vector. Such components include, for example, bacterial chaperone proteins such as SicA, GroEL, GroE, DnaK, CesT, SecB, FkpA, SkpA, etc.

One skilled in the art will appreciate that there are a wide variety of vectors which are suitable for expression in bacterial cells and which are readily obtainable. Vectors such as the pET series (Novagen, Madison, Wisconsin), the tac and trc series (Pharmacia, Uppsala, Sweden), pTTQ18 (Amersham International plc, England), pACYC 177, pGEX series, and the like are suitable for expression of a genomic streptavidin fusion protein. The choice of a host for the expression of a genomic streptavidin fusion protein is dictated in part by the vector. Commercially available vectors are paired with suitable hosts.

A wide variety of suitable vectors for expression in eukaryotic cells are also available. Such vectors include pCMVLaci, pXT1 (Stratagene Cloning Systems, La Jolla, California); pCDNA series, pREP series, pEBVHis, pDisplay (Invitrogen, Carlsbad, California). In certain embodiments, the genomic streptavidin fusion protein encoding nucleic acid molecule is cloned into a gene targeting vector, such as pMC1neo, a pOG series vector (Stratagene Cloning Systems).

As noted above, preferred host cells include, by way of example, bacteria such as *Escherichia coli*; mammalian cells such as Chinese Hamster Ovary (CHO) cells, COS cells, myeloma cells; yeast cells such as *Saccharomyces cerevisiae*;

insect cells such as *Spodoptera frugiperda*; plant cells such as maize, among other host cells.

Insect cells are capable of high expression of recombinant proteins. In this regard, baculovirus vectors, such as pBlueBac (see, e.g., U.S. Patent Nos. 5,278,050, 5,244,805, 5,243,041, 5,242,687, 5,266,317, 4,745,051 and 5,169,784; available from Invitrogen, San Diego, California) may be used for expression in insect cells, such as *Spodoptera frugiperda* Sf9 cells (see, U.S. Patent No. 4,745,051). Expression in insect cells or insects is preferably effected using a recombinant baculovirus vector capable of expressing heterologous proteins under the transcriptional control of a baculovirus polyhedrin promoter. (e.g., U.S. Patent No. 4,745,051 relating to baculovirus/insect cell expression system). Polyhedrin is a highly expressed protein, therefore its promoter provides for efficient heterologous protein production. The preferred baculovirus is *Autographa californica* (ACMNPV). Suitable baculovirus vectors are commercially available from Invitrogen.

Also, the fusion construct of the present invention may be expressed in transgenic animals. For example, the genomic streptavidin containing expression cassette may be operatively linked to a promoter that is specifically activated in mammary tissue such as a milk-specific promoter. Such methods are described in U.S. Patent No. 4,873,316 and U.S. Patent No. 5,304,498.

The genomic streptavidin gene fusion may also be expressed in plants, e.g., transgenic plants, plant tissues, plant seeds and plant cells. Such methods are described, e.g., in U.S. Patent No. 5,202,422.

Regardless of the particular system chosen, the design of systems suitable for expression of recombinant proteins is well known and within the purview of one of ordinary skill in the art, as evidenced by the above-identified references relating to expression of recombinant fusion proteins.

Accordingly, as is evidenced by the text and examples herein, expression of fusion proteins within the context of a genomic streptavidin expressed gene fusion construct provides several key advantages. For example, in one embodiment, the genomic streptavidin fusion protein is expressed as soluble protein into the periplasmic space of bacteria and undergoes spontaneous folding. Accordingly, such expression

offers the advantage that the periplasm is a low biotin, oxidizing environment and produces a soluble, functional molecule. This avoids having to purify and refold the protein under harsh denaturing conditions, which may prove fatal to the polypeptide encoded by the heterologous nucleic acid molecule.

5           The genomic streptavidin expressed gene fusion may be isolated by a variety of methods known to those skilled in the art. However, preferably the purification method takes advantage of the presence of a functional streptavidin molecule, by utilizing its high affinity binding to aid in purification. Accordingly, preferred purification methods are by the use of iminobiotin immobilized on a solid  
10 surface.

### C. ANTIBODIES AS FUSION COMPONENTS

While a broad variety of genomic streptavidin expressed gene fusion molecules may be designed by the methods described herein, a particularly useful fusion protein is that of an antibody and genomic streptavidin, in particular an  
15 antibody-genomic streptavidin expressed gene fusion (Ab-SA). In preferred embodiments the expression construct encodes an Fv or scFv portion of an antibody. In a further preferred embodiment the construct encodes a Fab fragment or functional derivative thereof, to which streptavidin may be linked via a terminus of either the heavy chain portion or light chain portion of the molecule. Accordingly, DNA  
20 encoding the Fv regions of interest may be prepared by any suitable method, including, for example, amplification techniques such as polymerase chain reaction from cDNA of a hybridoma, using degenerate oligonucleotides, ligase chain reaction (LCR) (see Wu and Wallace, *Genomics*, 4:560, 1989, Landegren *et al.*, *Science*, 241:1077, 1988 and Barringer *et al.*, *Gene*, 89:117, 1990), transcription-based amplification (see Kwoh *et*  
25 *al.*, *Proc. Natl. Acad. Sci. USA*, 86:1173, 1989), and self-sustained sequence replication (see Guatelli *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1874, 1990), cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang *et al.*, *Meth. Enzymol.* 68:90-99, 1979; the phosphodiester method of Brown *et al.*, *Meth. Enzymol.* 68:109-151, 1979; the  
30 diethylphosphoramidite method of Beaucage *et al.*, *Tetra. Lett.*, 22:1859-1862, 1981;

and the solid support method of U.S. Patent No. 4,458,066, as well as U.S. Patent Nos. 5,608,039 and 5,840,300, as well as PCT Application No. WO 98/41641. DNA encoding regions of interest, for example, Fab or scFv, may also be isolated from phage display libraries.

5 One of ordinary skill in the art would readily recognize that given the disclosure provided herein, any number of binding pair members may be utilized and thus would not be limited to streptavidin/biotin binding. In this regard, antibody/epitope pairs or any ligand/anti-ligand pair may be utilized. One of ordinary skill in the art would also appreciate that the present disclosure provides a general 10 method for the preparation of tetravalent antibodies. Since the avidity of an antibody for its cognate antigen is generally a function of its valency, there are many applications in which a tetravalent antibody would be preferable to a divalent antibody. Such applications include, but are not limited to, immunoassays, immunotherapy, immunoaffinity chromatography, etc.

15 Chemical synthesis may also be utilized to produce a single stranded oligonucleotide. This may be converted into double stranded DNA by hybridization with a complementary sequence, or by polymerization with a DNA polymerase using the single strand as a template. While it is possible to chemically synthesize an entire single chain Fv region, it is preferable to synthesize a number of shorter sequences 20 (about 100 to 150 bases) that are later ligated together.

Alternatively, subsequences may be cloned and the appropriate subsequences cleaved using appropriate restriction enzymes. The fragments may then be ligated to produce the desired DNA sequence.

Once the variable light ( $V_L$ ) and heavy chain ( $V_H$ ) DNA is obtained, the 25 sequences may be ligated together, either directly or through a DNA sequence encoding a peptide linker, using techniques well known to those of skill in the art. In a preferred embodiment, heavy and light chain regions are connected by a flexible polypeptide linker (e.g.,  $(Gly_4Ser)_x$ , or the pKOD sequence, or others, provided, *infra*) which starts at the carboxyl end of the light chain Fv domain and ends at the amino terminus of the 30 heavy chain Fv domain, or vice versa, as the order of the Fv domains can be either

light-heavy or heavy-light. The entire sequence encodes the Fv domain in the form of a single-chain antigen binding protein.

A variety of methods exist for the recombinant expression of immunoglobulins, the following references are representative of methods and host systems suitable for expression of recombinant immunoglobulins and fusion proteins in general: Weidle *et al.*, *Gene* 51:21-29, 1987; Dorai *et al.*, *J. Immunol.* 13(12):4232-4241, 1987; De Waele *et al.*, *Eur. J. Biochem.* 176:287-295, 1988; Colcher *et al.*, *Cancer Res.* 49:1738-1745, 1989; Wood *et al.*, *J. Immunol.* 145(a):3011-3016, 1990; Bulens *et al.*, *Eur. J. Biochem.* 195:235-242 1991; Beggington *et al.*, *Biol. Technology* 10:169, 1992; King *et al.*, *Biochem. J.* 281:317-323, 1992; Page *et al.*, *Biol. Technology* 9:64, 1991; King *et al.*, *Biochem. J.* 290:723-729, 1993; Chaudary *et al.*, *Nature* 339:394-397, 1989; Jones *et al.*, *Nature* 321:522-525, 1986; Morrison and Oi, *Adv. Immunol.* 44:65-92, 1988; Benhar *et al.*, *Proc. Natl. Acad. Sci. USA* 91:12051-12055, 1994; Singer *et al.*, *J. Immunol.* 150:2844-2857, 1993; Coto *et al.*, *Hybridoma* 13(3):215-219, 1994; Queen *et al.*, *Proc. Natl. Acad. Sci. USA* 86:10029-10033, 1989; Caron *et al.*, *Cancer Res.* 32:6761-6767, 1992; Dubel *et al.*, *J. Immunol. Methods* 178:201-209, 1995; Batra *et al.*, *J. Biol. Chem.* 265:15198-15202, 1990; Batra *et al.*, *Proc. Natl. Acad. Sci. USA*, 86:8545-8549, 1989; Chaudhary *et al.*, *Proc. Natl. Acad. Sci. USA*, 87:1066-1070, 1990, several of which describe the preparation of various single chain antibody expressed gene fusions.

Accordingly, once a DNA sequence has been identified that encodes an Fv region which when expressed shows specific binding activity, fusion proteins comprising that Fv region may be prepared by methods known to one of skill in the art. The Fv region may be fused to genomic streptavidin directly in the expression cassette of the present invention or, alternatively, may be joined directly to genomic streptavidin through a peptide or polypeptide linker, thereby forming a linked product. The linker may be present simply to provide space between the Fv and the fused genomic streptavidin or to facilitate mobility between these regions to enable them to each attain their optimum conformation. The genomic streptavidin-antibody expression cassette, typically, comprises a single vector which provides for the expression of both heavy and light variable sequences fused by an appropriate linker as well as a linker fusing the

light and heavy chains with genomic streptavidin, thereby encoding a single chain antibody:genomic streptavidin (scFvSA) conjugate. In one embodiment the linker connecting the variable light and heavy chains is of sufficient length or side group selection to allow for flexibility. In one embodiment the linker is a standard linker such 5 as (Gly<sub>4</sub>Ser)<sub>x</sub>, described *supra*, while in another embodiment the linker is the pKOD linker (GlyLeuGluGlySerProGluAlaGlyLeuSerProAspAlaGlySerGlySer) (SEQ ID NO: 9). It should be understood that a variety of linkers may be used, but in some 10 embodiments it may be preferred that the linker separating the light and heavy antibody chains should allow flexibility and the linker attaching the scFv to the genomic streptavidin sequence can be fairly rigid or fairly flexible. Further, in addition to 15 linkers, additional amino acids may be encoded by the addition of restriction sites to facilitate linker insertion and related recombinant DNA manipulation, as such these amino acids while not necessarily intended to be linkers may or may not be included within the constructs described herein, depending on the construction method utilized.

15                   Exemplary linkers are known by those of skill in the art. For example, Fv portions of the heavy and light chain of antibodies held together by a polypeptide linker can have the same binding properties as their full length two chain counterparts (Bird *et al.*, *Science*, 242:423-26, 1988 and Huston *et al.*, *Proc. Natl. Acad. Sci. USA*, 85:5879-83, 1988). It has also been shown that, in some cases, fusion proteins 20 composed of single chain antibodies linked to toxins may retain the binding capacity of the single chain antibody as well as the activity of the toxin (Chaudary *et al.*, *Nature*, 339: 394-97, 1989; Batra *et al.*, *J. Biol. Chem.*, 265: 15198-15202, 1990; Batra *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 8545-8549, 1989; Chaudary *et al.*, *Proc. Natl. Acad. Sci. USA* 87:1066-1070, 1990). Exemplary fusion constructs containing streptavidin 25 are described by Sheldon *et al.*, *Appl. Radiat. Isot.* 43(11):1399-1402, 1992; Sano and Cantor, *Bio/Technology* 9:1378-1381, 1991; Spooner *et al.*, *Human Pathology* 25(6):606-614, 1994; Dubel *et al.*, *J. Immun. Methods* 178:201-209, 1995; Kipriyanov *et al.*, *Protein Engineering* 9(2):203-211, 1996. The DNA sequence comprising the linker may also provide sequences, such as primer sites or restriction 30 sites, to facilitate cloning or may preserve the reading frame between the sequence

encoding the scFv and the sequence encoding genomic streptavidin. The design of such linkers is well known to those of skill in the art.

Further, one skilled in the art would find it routine to test the ability of genomic streptavidin-antibody expressed gene fusions to bind the appropriate ligand.

5 In contemplated embodiments, this ligand antigen may be a cell surface antigen, cell-associated stromal or matrix antigen, or cell-secreted antigens, including, but not limited to, CD19, CD20, CD22, CD25, CD33, CD45, CD52, CD56, CD57, EGP40 (or EPCAM or KSA), NCAM, CEA, TAG-72, a mucin (MUC-1 through MUC-7), -HCG, EGF receptor and variants thereof, IL-2 receptor, her2/neu, Lewis y, GD2, GM2, Lewis 10 x, folate receptor, fibroblast activation protein, tenascin, sialylated tenascin, somatostatin, activated tumor stromal antigen, or a neoangiogenic antigen. Moreover, methods for evaluating the ability of antibodies to bind to epitopes of such antigens are known.

**D. APPLICABLE USES OF GENOMIC STREPTAVIDIN EXPRESSED 15 FUSION CONSTRUCTS**

While any heterologous nucleic acid sequence can be joined to that encoding genomic streptavidin and expressed, as described herein, particularly useful expressed fusion constructs are those comprising scFv linked to genomic streptavidin, referred to previously as scFvSA. Accordingly, in one aspect of the invention, scFv fragments of antibodies are useful as tools in methods for medical diagnostic and therapeutic purposes. A diagnostic or therapeutic method, is described for detecting the presence or absence of, or treating, a target site within a mammalian host. When determining the criteria for employing antibodies or antibody conjugates for *in vivo* administration for therapeutic purposes, it is desirable that the generally attainable targeting ratio is high and that the absolute dose of therapeutic agent delivered to the tumor is sufficient to elicit a significant tumor response. Methods for utilizing such antibodies described in the present invention can be found, for example, in U.S. Patent Nos. 4,877,868, 5,175,343, 5,213,787, 5,120,526, and 5,200,169. Upon *in vivo* administration for therapeutic or diagnostic purposes, it is also desirable to limit the 30 exposure of non-target tissues to the therapeutic agent.

One method for reducing non-target tissue exposure to a diagnostic or therapeutic agent involves "pretargeting" the targeting moiety (*e.g.*, scFvSA) to a target site, and then subsequently administering a rapidly clearing diagnostic or therapeutic agent conjugate that is capable of binding to the "pretargeted" targeting moiety at the target site. In this method, as generally described, an optional intermediate step may involve administration of a clearing agent to aid in the efficient removal of the unbound targeting moiety conjugate from the circulation prior to administration of the active agent conjugate. A description of embodiments of the pretargeting technique, including the descriptions of various clearing agents and chelates, such as DOTA, may be found in U.S. Patent Nos. 4,863,713, 5,578,287, 5,608,060, 5,616,690, 5,630,996, 5,624,896; and PCT publication Nos. WO 93/25240, WO 95/15978, WO 97/46098, WO 97/46099, which are incorporated herein in their entirety.

In the pretargeting approach the pharmacokinetics of the active agent is decoupled from that of the targeting moiety. The targeting moiety, conjugated to a member of a ligand/anti-ligand pair, is permitted to accrete to target sites. Accordingly, in one embodiment of the present invention, scFvSA is a conjugate (fusion) of the targeting moiety (scFv) and ligand (streptavidin). After accretion occurs and a substantial fraction of the non-target associated conjugate is cleared from the recipient's circulation, either by intrinsic clearance or via administration of a ligand or anti-ligand containing clearing agent, the active agent is administered as a conjugate to the member of the ligand/anti-ligand pair that is complementary to that of the targeting conjugate (*e.g.*, biotin would be the complementary member in the exemplified embodiment). Preferably, the agent-ligand or agent-anti-ligand has a short serum half life and is excreted via the renal pathway. In this manner, the therapeutic agent either accretes to the target site where exertion of its therapeutic or diagnostic capability is desired, or it is rapidly removed from the recipient. This distribution of active agent facilitates the protection of normal tissues of the recipient from undesired toxicity. To enhance renal excretion, conjugation to a renal excretion-promoting biodistribution-directing molecule, may be employed. Essentially, such pretargeting methods are characterized by an improved targeting ratio or increased absolute dose to the target cell sites in comparison to conventional cancer diagnosis or therapy.

In one embodiment of the pretarget methodology, the targeting moiety will comprise an antibody fusion of the present invention specific for a particular antigen associated with the target cells of interest. In related embodiments the antigen marker may be associated with a cancer, including, but not limited to, the following:

5 lymphoma (e.g., CD20); leukemia (e.g., CD45); prostate (e.g., TAG-72); ovarian (e.g., TAG-72); breast (e.g., MUC-1); colon (e.g., CEA); and pancreatic (e.g., TAG-72). For example, the CD20 antigen may be targeted for the treatment of lymphoma wherein the ligand/anti-ligand binding pair may be biotin/avidin (e.g., streptavidin-gene fusion (scFvSA)), and the active agent will be a radionuclide in pretargeting methods. Further,

10 a variety of antigens may be targeted, such as CD45 antigen targeting for pretargeted radioimmunotherapy (PRIT) to treat patients having any one of a broad range of hematologic malignancies by employing antibody-mediated targeting to the CD45 antigen. CD45 is the most broadly expressed of the known hematopoietic antigens, found on essentially all white blood cells and their precursors, including neutrophils,

15 monocytes and macrophages, all lymphocytes, myeloid and lymphoid precursors, and about 90% of acute myelogenous leukemia (AML) cells. Accordingly, as the antigens available for targeting for diagnostic or therapeutic purposes are numerous, the present invention may be used to facilitate targeting to any of these antigens.

An optional step in pretarget methods, including those identified above,

20 comprises the initial administration of a non-conjugated targeting moiety (*i. e.*, not conjugated to a ligand or anti-ligand) or, alternatively, administering this non-conjugated targeting moiety concurrently with the conjugated form in the first step, thus blocking those targets contacted initially. Such blocking may be especially useful, for example, in the treatment of non-Hodgkin's lymphoma, where the first set of targeted

25 tissues may be the spleen, while most tumors are found in the deep lymph nodes. Such pre-blocking allows for substantial protection of the spleen cells from later treatment with the active agent. While the non-conjugated targeting agent need not necessarily bind the same epitope, to be effective it should preclude binding by the targeting moiety conjugate.

30 One skilled in the art could use multiple targeting moiety conjugates comprising different antibodies that also bind to the same cell type to enhance the

therapeutic effect or diagnostic utility. U.S. Patent No. 4,867,962 issued to Abrams describes such an improved method for delivering active agent to target sites, which method employs active agent-targeting moiety conjugates. Briefly, the Abrams method contemplates administration to a recipient two or more active agent-targeting moiety conjugates, wherein each conjugate includes a different antibody species as the targeting moiety. Each of the utilized antibody species is reactive with a different target site epitope (associated with the same, or a different, target site antigen), while the patterns of cross-reactivity of the antibody species with non-target tissues are non-overlapping. In this manner the different antibodies accumulate additively at the desired target site, while fewer than the total species accumulate at any type of non-target tissue. A higher percentage of the administered agent, therefore, becomes localized *in vivo* at target sites than at non-target tissues. The present invention encompasses approaches similar to this, as well as in pretargeting formats. In one embodiment, for example, two or more species of targeting conjugates (fusion) with antibodies directed to different epitopes and having non-overlapping cross-reactivity, each prepared according to the present invention, are administered according to the pretarget method so as to improve the diagnostic or therapeutic utility. A further embodiment utilizes the property that streptavidin monomers naturally associate to form tetramers. Thus, two or more antibodies each conjugated (fusion) to the monomeric form of streptavidin, are selectively combined and, upon formation of tetrameric streptavidin, yield single species with specificity for multiple epitopes at the target site.

It should be understood that the methods described may be modified and still achieve the desired effect. For example, two antibodies specific for the same antigen or cell type, regardless of their respective cross-reactivity, may be used. All that is necessary for these methods is that the targeting moiety-ligand/anti-ligand conjugate preferentially binds the target cells and that the active agent-conjugate substantially localizes to the pretargeted cells and is otherwise substantially cleared from circulation.

Alternatively, antibody-based or non-antibody-based targeting moieties may be employed to deliver a ligand/anti-ligand to a target site bearing an unregulated antigen. Preferably, a natural binding agent for such an unregulated antigen is used for this purpose.

Pretarget methods as described herein optionally include the administration of a clearing agent. The dosage of the clearing agent is an amount which is sufficient to substantially clear the previously administered targeting moiety-ligand/anti-ligand conjugate from the circulation. Generally, the determination of when

5 to administer the clearing agent depends on the target uptake and endogenous clearance of the targeting moiety conjugate. Particularly preferred clearing agents are those which provide for Ashwell receptor-mediated clearance, such as galactosylated proteins, e.g., galactosylated biotinylated human serum albumin, and small molecule clearing agents containing N-acetylgalactosamine and biotin.

10 Types of active agents (diagnostic or therapeutic) useful herein include radionuclides, toxins, anti-tumor agents, drugs, genes, and cytokines. For example, as described above, conjugates of such agents to biotin may be useful in the pretargeting approach. In this regard, a therapeutic antibody (e.g., an antibody that induces apoptosis or inhibits angiogenesis) may be used in a therapeutic modality such as 15 pretargeting. With regard to diagnostic agent fusions, in contrast to therapeutic agent fusions, enhanced target cell internalization is disadvantageous if one administers diagnostic agent-targeting moiety conjugates. Internalization of diagnostic conjugates results in cellular catabolism and degradation of the conjugate. Upon degradation, small adducts of the diagnostic agent or the diagnostic agent *per se* may be released 20 from the cell, thus eliminating the ability to detect the conjugate in a target-specific manner.

Diagnostic or therapeutic agents useful herein include radionuclides, drugs, anti-tumor agents, toxins, genes, and cytokines. Radionuclides useful within the present invention include gamma-emitters, positron-emitters, Auger electron-emitters, 25 X-ray emitters and fluorescence-emitters, with beta- or alpha-emitters preferred for therapeutic use. Radionuclides are well-known in the art and include  $^{123}\text{I}$ ,  $^{125}\text{I}$ ,  $^{130}\text{I}$ ,  $^{131}\text{I}$ ,  $^{133}\text{I}$ ,  $^{135}\text{I}$ ,  $^{47}\text{Sc}$ ,  $^{72}\text{As}$ ,  $^{72}\text{Se}$ ,  $^{90}\text{Y}$ ,  $^{88}\text{Y}$ ,  $^{97}\text{Ru}$ ,  $^{100}\text{Pd}$ ,  $^{101\text{m}}\text{Rh}$ ,  $^{119}\text{Sb}$ ,  $^{128}\text{Ba}$ ,  $^{197}\text{Hg}$ ,  $^{211}\text{At}$ ,  $^{212}\text{Bi}$ ,  $^{153}\text{Sm}$ ,  $^{169}\text{Eu}$ ,  $^{212}\text{Pb}$ ,  $^{109}\text{Pd}$ ,  $^{111}\text{In}$ ,  $^{67}\text{Ga}$ ,  $^{68}\text{Ga}$ ,  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ,  $^{75}\text{Br}$ ,  $^{76}\text{Br}$ ,  $^{77}\text{Br}$ ,  $^{99\text{m}}\text{Tc}$ ,  $^{11}\text{C}$ ,  $^{13}\text{N}$ ,  $^{15}\text{O}$ ,  $^{166}\text{Ho}$  and  $^{18}\text{F}$ . Preferred therapeutic radionuclides include  $^{188}\text{Re}$ ,  $^{186}\text{Re}$ ,  $^{203}\text{Pb}$ ,  $^{212}\text{Bi}$ ,  $^{213}\text{Bi}$ , 30  $^{109}\text{Pd}$ ,  $^{64}\text{Cu}$ ,  $^{67}\text{Cu}$ ,  $^{90}\text{Y}$ ,  $^{125}\text{I}$ ,  $^{131}\text{I}$ ,  $^{77}\text{Br}$ ,  $^{211}\text{At}$ ,  $^{97}\text{Ru}$ ,  $^{105}\text{Rh}$ ,  $^{198}\text{Au}$  and  $^{199}\text{Ag}$ ,  $^{166}\text{Ho}$  or  $^{177}\text{Lu}$ .

As one of ordinary skill in the art can readily appreciate the above streptavidin gene fusions may be utilized in combination therapies, such as when "pretargeting" is combined with the use of radiation-sensitizing agents. Such radiation sensitizing agents include, but are not limited to, gemcitabine, 5-fluorouracil, paclitaxel, 5 and the like.

Several of the potent toxins useful within the present invention consist of an A and a B chain. The A chain is the cytotoxic portion and the B chain is the receptor-binding portion of the intact toxin molecule (holotoxin). Because toxin B chain may mediate non-target cell binding, it is often advantageous to conjugate only 10 the toxin A chain to a targeting moiety (e.g., molecule). However, while elimination of the toxin B chain decreases non-specific cytotoxicity, it also generally leads to decreased potency of the conjugated toxin A chain, as compared to the conjugate of the corresponding holotoxin.

Preferred toxins in this regard include holotoxins, such as abrin, ricin, 15 modeccin, *Pseudomonas* exotoxin A, *Diphtheria* toxin, pertussis toxin, Shiga toxin, and bryotoxin; and A chain or "A chain-like" molecules, such as ricin A chain, abrin A chain, modeccin A chain, the enzymatic portion of *Pseudomonas* exotoxin A, *Diphtheria* toxin A chain, the enzymatic portion of pertussis toxin, the enzymatic portion of Shiga toxin, gelonin, pokeweed antiviral protein, saporin, tritin, barley toxin 20 and snake venom peptides. Ribosomal inactivating proteins (RIPs), naturally occurring protein synthesis inhibitors that lack translocating and cell-binding ability, are also suitable for use herein. Highly toxic toxins, such as palytoxin and the like, are also contemplated for use in the practice of the present invention. However, therapeutic drugs may themselves facilitate internalization of the complex.

25 Therapeutic drugs, administered as targeted conjugates, are also encompassed herein. Again, the goal is administration of the highest possible concentration of drug (to maximize exposure of target tissue), while remaining below the threshold of unacceptable normal organ toxicity (due to non-target tissue exposure). Unlike radioisotopes, however, therapeutic drugs need to be taken into a target cell to 30 exert a cytotoxic effect. In the case of targeting moiety-therapeutic drug conjugates, it would be advantageous to combine the relative target specificity of a targeting moiety

with a means for enhanced target cell internalization of the targeting moiety-drug conjugate.

Therapeutic drugs suitable for use herein include conventional chemotherapeutics, such as vinblastine, doxorubicin, bleomycin, methotrexate, 5-fluorouracil, 5 6-thioguanine, cytarabine, cyclophosphamide and cis-platinum, as well as other conventional chemotherapeutics including those described in *Cancer: Principles and Practice of Oncology*, 2d ed., V.T. DeVita, Jr., S. Hellman, S.A. Rosenberg, J.B. Lippincott Co., Philadelphia, Pennsylvania, 1985, Chapter 14, and analogues of such drugs where the analogue has greater potency than the parent molecule. Another drug 10 within the present invention is a trichothecene. Other preferred drugs suitable for use herein as a diagnostic or therapeutic active agent in the practice of the present invention include experimental drugs including those as described in *NCI Investigational Drugs, Pharmaceutical Data 1987*, NIH Publication No. 88-2141, Revised November 1987.

Other anti-tumor agents, *e.g.*, agents active against proliferating cells, 15 are administerable in accordance with the present invention. Exemplary anti-tumor agents include pro-apoptotic antibodies, anti-angiogenic antibodies, cytokines, such as IL-2, tumor necrosis factor or the like, lectin inflammatory response promoters (selectins), such as L-selectin, E-selectin, P-selectin or the like, and similar molecules.

One skilled in the art, based on the teachings in this application and the 20 applications referenced herein, can readily determine an effective diagnostic or therapeutic dosage and treatment protocol. This will depend upon factors such as the particular selected therapeutic or diagnostic agent, the route of delivery, the type of target site(s), affinity of the targeting moiety for the target site of interest, any cross-reactivity of the targeting moiety with normal tissue, condition of the patient, whether 25 the treatment is effected alone or in combination with other treatments, among other factors. A therapeutic effective dosage is one that treats a patient by extending the survival time of the patient. Preferably, the therapy further treats the patient by arresting the tumor growth and, most preferably, the therapy further eradicates the tumor.

All the references, including patents and patent applications, discussed 30 throughout, are hereby incorporated by reference in their entirety.

The present invention is further described through presentation of the following examples. These examples are offered by way of illustration and not by way of limitation.

## EXAMPLES

## EXAMPLE I

## CONSTRUCTION OF HUNR-LU-10 SINGLE CHAIN ANTIBODY-GENOMIC STREPTAVIDIN

## 5 FUSION

Generically, a single chain Fv/streptavidin (scFvSA) fusion protein is expressed from the genetic fusion of the single chain antibody of the variable regions (scFv) to the genomic streptavidin of *Streptomyces avidinii*. The scFv gene consists of 10 the variable regions of the light ( $V_L$ ) and heavy ( $V_H$ ) chains separated by a DNA linker sequence (e.g., Figure 2). The streptavidin coding sequence is joined to the 3' terminus of the scFv gene, and the two genes are separated in-frame by a second DNA linker sequence. The signal sequence from the streptavidin gene is fused at the 5' terminus of the scFvSA gene to direct expression to the *E. coli* periplasmic space. The scFvSA 15 gene is under control of the *lac* promoter, and the expressed fusion protein is extracted and purified from *E. coli* and forms a soluble tetramer of about 172,000 molecular weight.

Plasmid pKK233-2 (Amersham Pharmacia Biotech, Piscataway, NJ) was digested with *BamHI* and *NcoI* to remove the *trc* promoter. The *lac* promoter was 20 amplified from pBR322 by polymerase chain reaction (PCR) and cloned into the *BamHI/NcoI* site of pKK233-2. In the process an *EcoRI* site was introduced immediately 5' to the *NcoI* site. The plasmid was digested with *NcoI* and *PstI* and ligated with oligonucleotides encoding the *pelB* leader sequence. The accepting *NcoI* site on the plasmid was not regenerated and a new *NcoI* site was introduced in the 3' 25 area of the *pelB* encoding sequence. The resulting plasmid was referred to as pKK-lac/pelB (Figure 5). pKK-lac/pelB and pUC18 were digested with *PvuI* and *PvuII*. The 2.9 kb fragment of pKK-lac/pelB containing the *lac* promoter and multi-cloning site was ligated to the 1.4 kb fragment of pUC18 containing the origin of replication to form plasmid pEX-1 (Figure 6).

30 The streptavidin and huNR-LU-10 scFv genes (a monoclonal antibody that binds the antigen EGP40 or EPCAM, epithelial glycoprotein, 40 kD) were cloned

onto separate plasmids prior to construction of the huNR-LU-10 scFvSA gene. The streptavidin gene, signal sequence and approximately 300 bp of upstream sequence were PCR-amplified from *Streptomyces avidinii* (ATCC 27419) genomic DNA and cloned into pEX-1 as an *EcoRI/HindIII* fragment to form pEX318 (Figure 7). The huNR-LU-10 scFv was derived from the humanized antibody plasmid pNRX451 (Graves *et al.*, *Clin. Cancer Res.*, 5:899-908, 1999). The heavy and light chain variable regions were PCR-amplified separately from pNRX451 and then combined in a subsequent PCR. Oligonucleotides used in this process were designed to introduce a (Gly<sub>4</sub>Ser)<sub>3</sub> linker between the leading V<sub>L</sub> and the trailing V<sub>H</sub>. The resulting PCR product was cloned into pEX-1 as a *NcoI/HindIII* fragment forming the plasmid pEX-scFv3.2.1 (Figure 7). The scFv and streptavidin genes were PCR-amplified from pEX-scFv3.2.1 and pEX318, respectively, and combined into a fusion, as illustrated in Figure 8. The oligonucleotides used in these reactions created an overlap between the 3' end of the leading scFv and the 5' end of the trailing streptavidin, which encoded a five amino acid linker (GSGSA). The fragments were joined by PCR using the outside primers. The resulting 1.25 kb fragment was cloned into the *NdeI* and *BamHI* sites of vector pET3a (Novagen), generating pET3a-41B. This plasmid was digested with *XhoI* and *HindIII*, and the 1.3 kb fragment containing the V<sub>H</sub>-SA coding region and transcription terminator was ligated to a 4.6 kb *XhoI/HindIII* fragment of pEX-scFv3.2.1 containing the V<sub>L</sub> coding region, *lac* promoter, and ampicillin resistance gene (pYL256). The streptavidin regulatory region and signal sequence were PCR-amplified from pEX318 and cloned into the *EcoRI/NcoI* sites of pYL256 to form pEX94B (Figure 8).

The Tn5 kanamycin resistance gene (*neo*) was inserted into the huNR-LU-10 scFvSA expression plasmid pEX94B as follows (Figure 9): plasmid pNEO (Amersham Pharmacia) was digested with *BamHI*, blunt-ended with nucleotides using *Pfu* polymerase (Stratagene, La Jolla, CA), then further digested with *HindIII*. The 1494 bp fragment containing the kanamycin resistance gene was ligated to *HindIII/ScalI*-digested pEX94B plasmid, generating plasmid pEX94Bneo. The DNA sequence of the 1.6 kb *EcoRI* to *BamHI* fragment of plasmids pEX94B and pEX94Bneo is shown in Figure 10.

EXAMPLE II  
CONSTRUCTION OF B9E9 scFvSA FUSIONS

5           Additional single chain antibodies containing genomic streptavidin were constructed in a similar manner as noted above. A scFvSA version of the anti-CD20 mAb, B9E9, was constructed in the  $V_L V_H$  orientation with either a  $(\text{Gly}_4\text{Ser})_3$  (SEQ ID NO: 10)linker or a linker termed pKOD (amino acids GLEGSPEAGLSPDAGSGS) (SEQ ID NO: 9). Briefly, B9E9-1D3 hybridoma cells ( $1 \times 10^7$ )(from Bioprobe BV, 10 Amstelveen, The Netherlands) were harvested, and total RNA was prepared. The cDNAs for kappa chain and heavy chain of B9E9 were obtained by a reverse transcriptase reaction using primers RX207 and RX215, respectively. PCR fragments of variable regions of kappa chain and heavy chain were obtained using above cDNAs and pairs of oligos (RX207 and NX54 for kappa chain; RX215 and NX50 for heavy 15 chain). The PCR fragments were digested with *EcoRI* and *NotI* and subsequently cloned into a pPIC $\alpha$ A vector (Invitrogen, Sorrento Valley, CA), previously restricted with *EcoRI* and *NotI*. The resultant plasmids C58-1 and C58-16 carried B9E9 kappa chain and heavy chain, respectively. The two chains were further cloned out from C58-1 and C58-16 by PCR using pairs of oligos (RX468 and RX469 for kappa chain; 20 RX470 and RX471 for heavy chain). The kappa chain fragment was digested with *NcoI* and *BglII* and the heavy chain was digested with *XhoI-SacI*, respectively. The kappa chain was cloned into pEX94B (*NcoI-BglII*) as vector and heavy chain was cloned at *XhoI-SacI* sites in pEX94B. The resultant plasmids (C74-2 for kappa chain and C76-10 for heavy chain) were digested with *XhoI* and *HindIII*. The small fragment 25 from C76-10 was ligated into C74-2 vector restricted with the same enzymes. A resultant plasmid (C87-14) carried B9E9 scFvSA fusion protein with a  $(\text{G}_4\text{S})_3$  (SEQ ID NO: 10) linker between kappa chain and heavy chain. The C87-14 was further digested with *BglII* and *XhoI* and ligated with a pKOD linker prepared with two oligos (pInew5' and pInew3') to generate C136-1. Figures 11A and 11B illustrate the determined 30 nucleic acid sequence and predicted amino acid sequence of B9E9pKOD scFvSA.

Another version of B9E9 scFvSA was constructed in the  $V_H V_L$  orientation with an extended 25mer ( $Gly_4Ser$ )<sub>5</sub> (SEQ ID NO: 11) linker. The *NcoI-SacI* fragment of C87-14 containing scFv was further subcloned by PCR using a pair of primers (RX633 and RX471) to add a serine residue in the  $V_L$  region. The PCR fragment was digested 5 with *NcoI* and *SacI* and cloned into the pEX94B vector restricted with *NcoI* and *SacI*. The resultant plasmid D59-3 was subject to subcloning to generate the  $V_H$  or  $V_L$  fragments by PCR using RX781 and RX782 or RX729 and RX780, respectively. The  $V_H$  PCR fragment was digested with *NcoI* and *BglII* and cloned into the pEX94B vector 10 at the same sites to form D142-6. The  $V_L$  PCR fragment was digested with *XhoI* and *SacI* and cloned into the pEX94B vector at the same sites to form D142-1. A *XhoI-HindIII* fragment from D142-1 was isolated and replaced a *XhoI-HindIII* fragment of 15 D142-6 to generate D148-1 ( $V_H-V_L$  scFvSA). A *HindIII-BamHI* fragment, (blunted at *BamHI* side) containing a *neo* gene as described previously, was used to replace a *HindIII-ScaI* fragment of D148-1 to form D164-13. The D148-1 was also digested with 20 *BglII* and *XhoI* to remove the linker fragment and ligated with a 25mer linker (annealed with RX838 and RX839) to form E5-2-6. A *EcoRI-HindIII* fragment of E5-2-6 containing  $V_H-V_L$  scFvSA was excised and ligated with the D164-13 vector previously restricted with *EcoRI* and *HindIII* to form E31-2-20. Both plasmids E5-2-6 (carbenicillin-resistant) and E31-2-20 (kanamycin-resistant) express the B9E9 scFvSA fusion protein. Figure 11C illustrates the nucleic acid sequence and predicted amino acid sequence of B9E9 scFvSA ( $V_H-V_L$  25-mer).

All oligonucleotide primers, as listed below, were synthesized by Operon Technologies, Inc. (Alameda, CA).

5 NX50 (SEQ ID NO: 12)  
TGCCGTGAATTCGTSMARCTGCAGSARTCWGG

10 NX54 (SEQ ID NO: 13)  
TGCCGTGAATTCCATTSGCTGACCARTCTC

15 RX207 (SEQ ID NO: 14)  
TAGCTGGCGGCCGCCCTGTTGAAGCTTTGACAAT

20 RX215 (SEQ ID NO: 15)  
TAGCTGGCGGCCGCTTCTTGTCCACCTTGGTGC

25 RX468 (SEQ ID NO: 16)  
TTACGGCCATGGCTGACATCGTGCTGCAGTCTCCAGCAATCCTGTCT

30 RX469 (SEQ ID NO: 17)  
CACCAGAGATCTTCAGCTCCAGCTTGGTCCC

35 RX470 (SEQ ID NO: 18)  
CGGAGGCTCGAGCCAGGTTCAGCTGGCCAGTCAGGGCTGAGCTGGTGAAG

40 RX471 (SEQ ID NO: 19)  
GAGCCAGAGCTCACGGTACCGTGGTCCCTGCGCCCC

45 pInew5' (SEQ ID NO: 20)  
GATCTCTGGTCTGGAAGGCAGCCCGGAAGCAGGTCTGTCTCCGGACGCAGGTTCCGGC

50 pInew3' (SEQ ID NO: 21)  
TCGAGCCGGAACCTGCGTCCGGAGACAGACCTGCTTCCGGCTGCCCTCCA

55 GACCAGA

60 RX633 (SEQ ID NO: 22)  
TTACGGCCATGGCTGACATCGTGCTGCGAGTCTCCAGCAATCCTGTCT

65 RX779 (SEQ ID NO: 23)  
TTCCGGCTCGAGCGACATCGTGCTGCGAGTCTCCA

70 RX780 (SEQ ID NO: 24)  
GAGCCAGAGCTTCAGCTCCAGCTTGGTCCC

75 RX781 (SEQ ID NO: 25)

TTACGGCCATGGCTCAGGTTCAGCTGGTCCAGTCA

5 RX782 (SEQ ID NO: 26)

AGACCAGAGATCTGCTACGGTACCGTGGTCCC

10 RX838 (SEQ ID NO: 27)

GATCTCTGGTGGCGGTGGCTCGGGCGGTGGTGGGTGGGTGGCGGCCGGCTC  
GGGTGGTGGTGGGTGGCTCGGGCGGCAGCAGC

15 RX839 (SEQ ID NO: 28)

TCGAGCCGCCGCCGCCGACCCACCACCCGAGCCGCCACCCGACC  
CACCACCGCCCCGAGCCACCGCCACCAGA

15

### EXAMPLE III

#### EXPRESSION OF HUNR-LU-10 scFvSA AND B9E9 scFvSA PROTEINS

Transformants of *E. coli* strain XL1-Blue (Stratagene, La Jolla, CA) containing plasmids pEX94B (huNR-LU-10 scFvSA) or E5-2-6 (B9E9 scFvSA) were grown overnight at 30°C in Terrific broth (20 ml; Sigma) containing carbenicillin (50 µg/ml). The culture was diluted 100-fold into fresh medium and grown in a shaking incubator at 30°C. When the culture attained an  $A_{600}$  of 0.3-0.5, IPTG (Amersham Pharmacia Biotech, Piscataway, NJ) was added to a final concentration of 0.2 mM, and incubation was continued overnight. Periplasmic extracts were prepared for qualitative analysis of the scFvSA expression level. Cells were resuspended in an ice-cold solution of 20% sucrose, 2 mM EDTA, 30 mM Tris, (pH 8.0), and lysozyme (2.9 mg/ml) and were incubated on ice for 30 min. Supernatants were analyzed on 4-20% Tris-glycine SDS-PAGE gels (Novex) under non-reducing, non-boiled conditions, and gels were stained with Coomassie Blue. Expression in shake flasks was optimized by testing different environmental parameters, such as IPTG concentration and timing, temperature, media, or carbon source, or testing genetic factors, such as different promoters or signal sequences.

Clones were further grown in an 8L fermentor and analyzed for expression level. The primary inoculum (50 ml) was grown overnight at 30°C in shake flasks containing Terrific broth plus 50 µg/ml kanamycin (plasmids pEX94Bneo or

E31-2-20) or carbenicillin (plasmids pEX94B or E5-2-6), depending on the selectable marker of the plasmid. The culture was then diluted 100-fold into the same medium and grown at 30°C for an additional 4-5 h. This secondary inoculum (0.5 liter) was transferred to a 14 liter BioFlo 3000 fermentor (New Brunswick Scientific) containing

5 8 liters of complete *E. coli* medium [per liter: 6 g Na<sub>2</sub>HPO<sub>4</sub>, 3 g KH<sub>2</sub>PO<sub>4</sub>, 0.5 g NaCl, 3 g (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>, 48 g yeast extract (Difco), 0.25 ml Mazu DF204 antifoam (PPG Industries Inc., Pittsburgh, PA), 0.79 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 0.044 g CaCl<sub>2</sub> · 2H<sub>2</sub>O, and 3 ml of trace elements (per liter: 0.23 g CoCl<sub>2</sub>, 0.57 g H<sub>3</sub>BO<sub>3</sub>, 0.2 g CuCl<sub>2</sub> · 2H<sub>2</sub>O, 3.5 g FeCl<sub>3</sub> · 6H<sub>2</sub>O, 4.0 g MnCl<sub>2</sub> · 4H<sub>2</sub>O, 0.5 g ZnCl<sub>2</sub>, 1.35 g thiamine, and 0.5 g Na<sub>2</sub>MoO<sub>4</sub> · 2H<sub>2</sub>O)]. The

10 medium contained an initial 5 g/liter galactose as carbon source plus 50 µg/ml of kanamycin or carbenicillin for plasmid retention. The culture was grown at 30°C and induced with IPTG (0.2 mM) at 6 h post-inoculation. The pH was maintained at 7.0 by the automatic addition of either phosphoric acid or NaOH. Dissolved oxygen concentration was maintained at or above 30% throughout the run using agitation

15 speeds of 400-800 rpm and oxygen supplementation as necessary. A galactose solution (50%) was fed over a 9 h period after exhaustion of the initial galactose present in the medium to a total of 20-25 g per liter. Cells were harvested at 24-26 h post-inoculation (for B9E9 scFvSA) or 48-56 h post-inoculation (for huNR-LU-10 scFvSA) in a continuous flow centrifuge (Pilot Powerfuge, Carr Separations, Franklin, MA), washed

20 with PBS (10 mM sodium phosphate, 150 mM NaCl, pH 7.2), and pelleted by centrifugation. A typical fermentation produced 80-90 g of cells (wet wt) per liter culture medium.

For determining expression levels, cells were washed twice in PBS, resuspended to the original volume, and disrupted either by sonication on ice (Branson 25 Ultrasonics, Danbury, CT) or through two cycles of microfluidization (Microfluidics International, Newton, MA). Two assays were used for quantitating fusion protein in the supernatant of a centrifuged sample of crude lysate. Initially, an ELISA assay was used in which biotinylated albumin (100 ng per well in PBS) was coated overnight in 96-well plates at 4°C and incubated with serial two-fold dilutions of either HPLC-purified fusion protein (200 ng/ml) or test samples. Detection was using peroxidase-labeled goat anti-streptavidin polyclonal antibody (Zymed, So. San Francisco, CA) and

ABTS (Sigma) substrate buffer. Plates were read at 415/490 nm with a dual wavelength automated plate reader. A first order, log x/log y regression analysis was performed for quantitation of the fusion protein.

Alternatively, a rhodamine-biotin HPLC assay was devised that 5 provided faster results. The fusion protein in centrifuged lysates was complexed with excess rhodamine-derivatized biotin, which was prepared as follows: 5-(and-6)-carboxytetramethylrhodamine, succinimidyl ester (Molecular Probes, Eugene OR) was coupled to biocytin (Pierce, Rockford IL) through the formation of a stable amide bond. The reaction mixture was purified by HPLC using a Dynamax semi-preparative C-18 10 column (Rainin Instrument Co., Woburn, MA). The effluent was monitored at 547 nm and peak fractions collected and analyzed by mass spectrometry. Fractions corresponding in molecular weight to biocytin-rhodamine conjugate were pooled and concentrated by roto-evaporation (Buchii, Switzerland). An excess of purified biocytin-rhodamine conjugate was added to the clarified crude lysate and analyzed by 15 size exclusion chromatography using a Zorbax GF-250 column (MAC-MOD, Chadds Ford PA) equilibrated in 20 mM sodium phosphate containing 15% DMSO at 1.0 ml/min flow rate. The effluent was monitored at 547 nm using a Varian Dynamax PDA-2 detector, and the peak area corresponding to fusion protein elution was determined using a Varian Dynamax HPLC Data System (Walnut Creek, CA). The 20 concentration of fusion protein in the crude lysate was calculated by comparison to a standard analyzed under the same conditions. The molar extinction coefficient for the fusion protein standard was calculated using a previously described method summing the relative contributions of amino acids absorbing at 280 nm (Gill and von Hippel, *Analyt. Chem.* 182:319-326, 1989).

25 Expression levels in fermentor-grown cells were 100-130 mg/liter for huNR-LU-10 scFvSA, 40 mg/liter for B9E9pKOD scFvSA, and 270-300 mg/liter for B9E9 scFvSA ( $V_H$ - $V_L$  25-mer).

## EXAMPLE IV

## EXPRESSION OF B9E9 scFvSA USING VARIOUS LINKERS AND SIGNAL SEQUENCES

5 A number of genetic variants were constructed that contained linkers of different lengths and composition and the variable regions in different order (Table 1). These constructs were initially grown and induced in shake flask cultures and qualitatively assessed for expression by visualizing periplasmic proteins on Coomassie-stained, non-reducing, SDS gels. High-expressing constructs were further tested in an  
 10 8L fermentor using a galactose fed-batch protocol, and their expression levels were quantitatively determined by size exclusion HPLC using rhodamine-derivatized biotin. The construct that best fulfilled these criteria contained a 25-mer Gly<sub>4</sub>Ser linker with the scFv in the V<sub>H</sub>V<sub>L</sub> orientation.

15

**Table 1.** Summary of expression levels of B9E9 scFvSA genetic variants.

| V <sub>L</sub> -V <sub>H</sub> -SA |                         | V <sub>H</sub> -V <sub>L</sub> -SA |            |
|------------------------------------|-------------------------|------------------------------------|------------|
| Linker type <sup>a</sup>           | Expression <sup>b</sup> | Linker type                        | Expression |
| 15 mer G4S                         | +                       | 15 mer G4S                         | 60 mg/L    |
| 18 mer G4S                         | ++                      | 18 mer G4S                         | 195 mg/L   |
| 25 mer G4S                         | ++                      | 25 mer G4S                         | 300 mg/L   |
| 35 mer G4S                         | ++                      |                                    |            |
| 18 mer pKOD                        | 40 mg/L                 | 18 mer pKOD                        | 60 mg/L    |
| 18 mer pKOD2                       | ++                      | 18 mer pKOD2                       | +++        |

<sup>a</sup> Linker sequences (L1):

20 15 mer G4S: GGGGSGGGGGGGGG (SEQ ID NO: 10)  
 18 mer G4S: GGGGSGGGGGGGGGGG (SEQ ID NO: 29)  
 25 mer G4S: GGGGSGGGGGGGGGGGGGGG (SEQ ID NO: 11)  
 35 mer G4S: 7 copies of GGGG (SEQ ID NO: 30)  
 18 mer pKOD: GLEGSPEAGLSPDAGSGS (SEQ ID NO: 9)  
 25 18 mer pKOD2: GLEGSPEAGLSPDAGSDS (SEQ ID NO: 31)

<sup>b</sup> The expression levels of the fusion proteins were based on 8L fermentor runs except for those qualitative data, designated as plus symbols, based on SDS-PAGE analysis of shake-flask cultures.

## EXAMPLE V

INCREASED EXPRESSION OF SCFVSA FUSION PROTEINS IN PERIPLASM OF *E. COLI*

The *E. coli* *fkpA* gene is a member of the family of FK506-binding proteins (FKBPs) and is one of the periplasmic components involved in protein folding. It is expressed in the *E. coli* periplasm and has peptidyl-prolyl isomerase (PPIase) activity. The PPIase-independent chaperone activity of the FkpA gene product has also been demonstrated both *in vivo* and *in vitro*. The FkpA chaperone protein is involved in a protein-folding process by stabilizing the folding intermediates in the periplasm. It was tested whether co-expression of the single chaperone gene (*fkpA*) was able to stimulate the expression of scFvSA fusion proteins, especially among those that had not previously expressed well in *E. coli*.

In order to clone the DNA fragment of the *fkpA* gene, chromosomal DNA was extracted from *E. coli* XL1-Blue cells (Stratagene) and digested with *Xba*I. Thirty-five cycles of PCR were performed using a pair of oligonucleotides (RX1229: ACGACGGTTGCTGCGGCGGTC (SEQ ID NO: 32); RX1231: AGGCTCATTAAAT GATGCGGGT (SEQ ID NO: 33); both obtained from Operon Technologies, Inc.) and 300 ng of the digested genomic DNA as a template. The PCR mixture was subject to a second round of PCR (30 cycles) using a pair of nested oligonucleotides (RX1230: GGATCCAAGCTTACGATCACGGTCATGAACACG (SEQ ID NO: 34); RX1232: CTCGAGAAGCTTAACTAAATTAAATACAGCGGA) (SEQ ID NO: 35). The PCR fragments were resolved on a 1% agarose gel, and the 1.6-kb fragment was isolated. The extracted DNA was cloned into the TA vector (Invitrogen), and the sequence was confirmed by DNA sequencing. The clone was digested with *Hind*III, using a site that was incorporated into oligonucleotides RX1230 and RX 1232 and was ligated with *Hind*III-digested vector E84-2-8 (NeoRx Corp.), harboring the anti-CEA T84.66 scFvSA fusion gene (T84.66 cDNA from City of Hope, Duarte, CA). The resultant plasmid (F115-1-1) was used to transform XL1-Blue *E. coli* for shake-flask expression. The periplasmic components were extracted and analyzed on 4-20% SDS-PAGE. For electrophoretic analysis, 20  $\mu$ l of the solution of scFvSA periplasmic fusion proteins

were loaded in each lane of the gel. Following electrophoresis, the gel was stained with Coomassie Blue R250. The FkpA protein, with a molecular weight of about 30,000, was prominently present in all samples carrying the *fkpA* gene (+), while absent in those lacking the gene (-), as shown, for example, in Figure 19. The molecular weights of the 5 seven components in the SeeBlue molecular standard marker (M), obtained from Novex, listed in order of increasing size, from the bottom of the gel, are 16,000; 30,000; 36,000; 50,000; 64,000; 98,000; and 250,000. As seen in Figure 19, expression of the T84.66 scFvSA fusion protein increased dramatically when co-expressed with the FkpA chaperone protein, in comparison to the parent construct (E84-2-8) lacking 10 the *fkpA* gene. Additional scFvSA fusions were constructed by moving *NcoI-SacI* fragments to the F115-1-1 vector, which had previously been restricted with *NcoI* and *SacI*. The resultant plasmids were tested in *E. coli* XL1-Blue shake flask cultures. Upon electrophoretic analysis, several showed increased fusion protein expression, as demonstrated in Figure 19 and Table 2. The results summarized here involve only the 15  $V_h$ - $V_l$ -SA fusion configuration incorporating the (Gly<sub>4</sub>Ser)<sub>5</sub> (SEQ ID NO: 11)linker. As summarized in Table 2, the expression levels of fusion proteins in the shake flask experiments were estimated qualitatively, with the highest level assigned a level of +++++.

20 **Table 2.** Qualitative expression of scFvSA fusion proteins in *E. coli*.

| <i>Antigen</i> | <i>scFvSA</i> | SEQ ID NO. | <i>Expression level</i> |                   |
|----------------|---------------|------------|-------------------------|-------------------|
|                |               |            | FkpA <sup>-</sup>       | FkpA <sup>+</sup> |
| <i>CEA</i>     | T84.66        | 36         | -                       | ++                |
|                | Col-1         | 37         | -                       | +                 |
|                | PR1A3         | 38         | -                       | -                 |
|                | MFE-23        | 39         | ++++                    | ++                |
|                | Nrco-2        | 40         | +++                     | +                 |
| <i>Tag-72</i>  | CC49          | 41         | ++                      | ++++              |
| <i>MUC-1</i>   | BrE-3         | 42         | -                       | -+                |
| <i>c-erbB2</i> | ICR12         | 43         | -                       | -                 |
| <i>CD20</i>    | B9E9          | 44         | +++                     | +++++             |
|                | C2B8          | 45         | -                       | -                 |
| <i>CD45</i>    | BC8           | 46         | +                       | +++               |

EXAMPLE VI  
PURIFICATION OF HUNR-LU-10 scFvSA AND B9E9 scFvSA PROTEINS

5

The iminobiotin affinity matrix was prepared by reacting epoxide-activated Macro-prep matrix (BioRad, Hercules CA) with 112  $\mu$ m N-(3-amino-propyl)-1,3 propane diamine (Sigma) per g of matrix in 0.2 M carbonate buffer. The reaction 10 was stopped after 8 h by filtering the slurry through a scintered glass funnel and rinsing the matrix with distilled water. Residual epoxides were inactivated by reacting the matrix with 0.1 M sulfuric acid for 4 h at 80°C, and the matrix was again rinsed. The amine-derivitized matrix was suspended in PBS, and the pH increased to 8.5 by the addition of 10% volume of 0.5 M sodium borate, pH 8.5. NHS-iminobiotin (Pierce) 15 was dissolved in DMSO and added to the suspended matrix at a ratio of 2.6 mg/g of matrix. Following a 4 h reaction, the matrix was rinsed with distilled water followed by several alternating washes with pH 11 sodium carbonate buffer and pH 4 sodium acetate buffer and a final rinse with distilled water. The matrix was stored as a slurry in 20% ethanol.

20

Cells (650-750 g, wet wt) were washed twice in PBS, resuspended to 10-20% weight per volume with ice-cold 30 mM Tris, 1 mM EDTA, pH 8, and disrupted through two cycles of microfluidization. The lysate was adjusted to 50 mM glycine, 450 mM NaCl, pH 9.6, with a conductivity range of 46-48 mSe per cm, and then centrifuged at 12,000 rpm for 90 min. The supernatant was filtered (0.2 m), then 25 affinity purified over immobilized iminobiotin. The iminobiotin matrix was packed in a column and equilibrated in 50 mM glycine, 500 mM NaCl, pH 9.6 with a conductivity of 46-48 mSe per cm. Capacity using recombinant streptavidin (Roche Biochemical, Indianapolis, IN) was 2 mg per ml of bed volume under a flow of 2 ml/cm<sup>2</sup>/min. The 0.2 m filtered cell homogenized supernatant was pumped at room 30 temperature at 2 ml/cm<sup>2</sup> per min using 80 ml of bed volume per 100 g of cells. After washing with 20 bed volumes of column equilibrating buffer, the scFvSA fusion

protein was eluted with 0.2 M sodium acetate, 0.1 M NaCl, pH 4.0, neutralized with Tris buffer, and then exhaustively dialyzed in refrigerated PBS.

To reduce protein aggregation, purified scFvSA was treated with 10% DMSO for 5-7 h at room temperature and dialyzed in PBS. The purified protein was 5 concentrated using an Amicon YM30 membrane apparatus and filter-sterilized for aseptic storage at 4°C. At concentrations of 2-3 mg/ml, purified preparations typically contained ca. 5-8% aggregate.

Typical recoveries from iminobiotin chromatography were 50-60% with less than 5% appearing in the flow-through and wash. The residual remained as 10 aggregate/entrapped material on the column. Addition of DMSO to the eluting buffer yielded <5% additional purified protein. Use of a variety of ionic and nonionic detergents did not improve recoveries. HPLC size exclusion analysis of the eluted fusion protein showed that up to 40% of the protein was in an aggregated form. Light scattering HPLC indicated aggregate sizes between 400,000 and 4 million. Treatment 15 with 10% DMSO for several hours resulted in the slow de-aggregation of the fusion protein, yielding >92% tetrameric species that remained so when stored refrigerated in PBS at a concentration of <3 mg/mL.

20

#### EXAMPLE VII

#### BIOCHEMICAL CHARACTERIZATION OF HUNR-LU-10 SCFvSA AND B9E9 SCFvSA PROTEINS

**SDS-PAGE Analysis.** Purified fusion proteins were analyzed on 4-20% 25 Tris-glycine SDS-PAGE gels (Novex, San Diego, CA) under nonreducing conditions. Before electrophoresis, samples were mixed with SDS-loading buffer and incubated at either room temperature or 95°C for 5 min. Gels were stained with Coomassie blue.

SDS-PAGE demonstrated that the fusion proteins were purified to > 95% homogeneity after iminobiotin chromatography (Figure 12, lanes 2 & 3; huNR-LU-10 data only). The major band migrated at the expected molecular weight of ~173 kDa with minor isoforms evident. These isoforms were also detected with polyclonal 5 anti-streptavidin antibody on Western gel analysis (data not shown). However, all bands resolved into a single species of ~ 43 kDa when the protein was boiled prior to electrophoresis, consistent with a single protein entity dissociable into its homogeneous subunit (Figure 12, lanes 4 & 5). The molecular weights of the seven components in the SeeBlue molecular standard marker (Figure 12, lane 1), available from Novex, are 10 described in Example V.

**Size exclusion HPLC and Laser Light Scattering Analysis.** Purified protein preparations were analyzed by size exclusion HPLC performed on a Zorbax GF-250 column with a 20 mM sodium phosphate/0.5 M NaCl mobile phase. The 15 molecular weight of the fusion construct was measured using this Zorbax system connected in series with a Varian Star 9040 refractive index detector and a MiniDawn light scattering instrument (Wyatt Technologies, Santa Barbara, CA). A dn/dc value of 0.185 for a protein in an aqueous buffer solution was used in the molecular weight calculations.

20 HPLC size exclusion chromatography exhibited a major peak with a retention time appropriate for the huNR-LU-10 tetramer with a minor (<8%) aggregate peak (Figure 13). B9E9 scFvSA showed a very similar profile (graph not shown). These analyses demonstrated that all of the purified protein was tetrameric or an aggregate thereof. Light scattering analysis of huNR-LU-10 scFvSA indicated a 25 molecular weight of 172,600, as predicted for the tetrameric protein.

**Amino-terminal sequencing.** Automated amino acid sequencing was performed using a Procise 494 sequenator (Applied Biosystems, Inc., Foster City, CA). This revealed that the leader sequences of both huNR-LU-10 scFvSA and B9E9 30 scFvSA were cleaved at the expected signal peptidase site adjacent to the first amino acid of the variable region.

**Molecular weight determination of B9E9 scFvSA.** Liquid chromatographic separation was conducted with an Hewlett Packard series 1100 system, fitted with a Jupiter C18 column (300 Å, 3.2 x 50 mm, 5 µ) and C18 "SafeGuard" column (Phenomenex, Torrance, CA) at a flow rate of 500 µl/min. The 5 mobile phase was composed of water/1% formic acid (buffer A) and acetonitrile/1% formic acid (buffer B). The gradient applied was 2% B for 3 min rising to 99% B within 7 min. B9E9 scFvSA (10 µl) was eluted at a retention time of 8.7 min. The analytical column was interfaced with a Thermoquest/Finnigan ESI LCQ ion trap mass spectrometer (San Jose, CA). The instrument was calibrated with myoglobin and 10 operated in the positive ion mode with the heated capillary set to 200°C and 5.1 kV applied to the electrospray needle. The data were acquired in a full scan MS mode (*m/z* [500-2000 Da/z]) using automated gain control with 3 microscans and a maximum ion time of 500 ms.

15 The mass spectrum of the B9E9 monomer showed a deconvoluted molecular weight of 43,401, which is in agreement with the calculated most abundant mass of 43,400.

**HuNR-LU-10 Competitive Immunoreactivity ELISA.** Serial dilutions of the humanized NR-LU-10 whole antibody or the huNR-LU-10 fusion protein were 20 allowed to compete with peroxidase-labeled murine NR-LU-10 whole antibody for binding to an 0.1% NP40 membrane extract from the human carcinoma cell line, LS-174 (ATCC #CL188). Following a log-logit transformation of the data in which curves were fit to the same slope, the concentration of competitor antibody that gave 50% inhibition (k) was calculated. Percent immunoreactivity was determined according the 25 formula: k (fusion protein standard)/k (whole antibody standard) x 100. The huNR-LU-10 fusion protein was found to possess immunoreactivity superior (~225%) to the intact divalent humanized antibody (Figure 14).

**B9E9 Competitive Immunoreactivity FACS Assay.** Immunoreactivity 30 was assessed in a competitive binding assay using flow cytometry that measured the binding of fluorescein-labeled B9E9 to the CD20-positive Ramos cell line (Burkitt's

lymphoma; ATCC CRL-1596) in the presence of various concentrations of unlabeled antibody. B9E9 mAb was labeled using fluorescein N-hydroxysuccinimide, and an optimized amount of this conjugate was mixed with serial dilutions (3-200 ng/ml) of B9E9 mAb standard or molar equivalents of B9E9 scFvSA and incubated with  $1 \times 10^6$  cells at 4°C for 30 minutes. Samples were washed and then analyzed on a single laser FACSCalibur (Becton Dickinson). After gating on single cells, the geometric mean fluorescence intensity was determined from a histogram plot of fluorescence. The concentration of competitor antibody required for 50% inhibition ( $IC_{50}$ ) of fluorescein-B9E9 binding was calculated using nonlinear regression analysis for one-site binding.

10 Percent immunoreactivity =  $[IC_{50} \text{ scFvSA} / IC_{50} \text{ mAb}] \times 100$ .

The scFvSA was about twice as immunoreactive (~185%) as the divalent B9E9 antibody on a molar basis, and nearly equivalent (~93%) to B9E9 mAb when adjusted for tetravalency (graph not shown).

15 **B9E9 scFvSA Avidity.** Avidity was determined using saturation binding experiments that measure specific binding of radiolabeled mAb or fusion protein (0.025-50 ng/ml) at equilibrium in the presence of excess antigen ( $10^7$  cells). Nonspecific binding was determined in the presence of excess cold mAb or fusion protein (50  $\mu$ g/ml). Mixtures were incubated and centrifuged as described above. The 20 equilibrium dissociation constant ( $K_d$ ) was calculated from nonlinear regression analysis of nM bound vs. nM radioligand using immunoreactivity-adjusted antibody concentrations. The B9E9 fusion protein retained the same relative nanomolar avidity as the B9E9 mAb, as determined by radiolabeled binding to Ramos cells (Table 3).

25 **Table 3.** Avidity of B9E9 mAb and scFvSA fusion protein.

| Antibody             | $K_d$ (nM) <sup>a</sup> | $K_a$ ( $\times 10^8 \text{ M}^{-1}$ ) |
|----------------------|-------------------------|----------------------------------------|
| B9E9 mAb             | 9.75                    | 1.02                                   |
| B9E9 scFvSA (25-mer) | 12.44                   | 0.80                                   |

<sup>a</sup>Antibody concentrations were adjusted for immunoreactivity (67% and 79% for mAb and scFvSA, respectively).  $K_d$  and  $K_a$  were calculated using nonlinear regression analysis of nM bound vs. nM radioligand.

**Biotin Binding and Dissociation.** Biotin binding capacity was determined by incubation of a known quantity of fusion protein with a 9-fold molar excess of [<sup>3</sup>H]biotin (NEN Research Products, Boston, MA). After removal of 5 uncomplexed biotin using streptavidin-immobilized beads (Pierce Chemical; Rockford, IL), the amount of [<sup>3</sup>H]biotin associated with the fusion protein was determined.

HuNR-LU-10 scFvSA and B9E9 scFvSA were capable of binding an average of 3.0 and 3.6 biotins, respectively, as compared to 4 biotin binding sites for recombinant streptavidin.

10 For huNR-LU-10 scFvSA, the rate of DOTA-biotin dissociation was assessed at 37°C in 0.25 M phosphate, 0.15 M sodium chloride, pH 7.0 containing either 10  $\mu$ M fusion protein or recombinant streptavidin and a subsaturating level of [<sup>90</sup>Y]DOTA-biotin. A 100-fold saturating level of biocytin (Sigma) was added to initiate the dissociation measurement. At timed intervals, aliquots of incubate were 15 diluted in PBS containing 0.5% bovine serum albumin. In order to precipitate the protein, zinc sulfate was added to each diluted aliquot, followed by sodium hydroxide, each to yield a final concentration of 0.06 M. Following microcentrifugation, free [<sup>90</sup>Y]DOTA-biotin in the supernatant was assessed using a Hewlett Packard beta counter. The DOTA-biotin dissociation rate of huNR-LU-10 scFvSA was comparable 20 to that of recombinant streptavidin ( $t_{1/2}$  of 58 min for huNR-LU-10 scFvSA vs. 47 min for recombinant streptavidin; Figure 15).

25 For B9E9 scFvSA, biotin dissociation was measured as described above, except [<sup>3</sup>H]biotin was used instead of [<sup>90</sup>Y]DOTA-biotin. The calculated  $t_{1/2}$  for biotin dissociation was 379 min for B9E9 scFvSA vs. 364 min for recombinant streptavidin (graph not shown).

#### EXAMPLE VIII

##### ANALYSIS OF BIODISTRIBUTION OF <sup>111</sup>IN-DOTA-BIOTIN AFTER PRETARGETING WITH

The expressed huNR-LU-10 scFvSA gene fusion was tested in a full pretarget protocol in female nude mice bearing SW-1222 human colon cancer xenografts (100-200 mg), subcutaneously implanted on the right flank. In these experiments, 575 µg of <sup>125</sup>I-labeled fusion protein was injected intravenous (iv) and 5 allowed to circulate for 18 hours prior to iv injection of 100 µg of synthetic clearing agent (sCA) (See e.g., PCT Publication Nos. WO 97/46098 and WO 95/15978). Three hours after the sCA injection, there was an injection of 1.0 µg of <sup>111</sup>In-DOTA-biotin, essentially a chelating agent containing a radionuclide, conjugated to biotin (see U.S. Patent Nos. 5,578,287 and 5,608,060). Mice were sampled for blood, then sacrificed 10 and dissected at 2, 24, 48, and 120 hours after <sup>111</sup>In-DOTA-biotin injection.

The concentration of <sup>125</sup>I-huNR-LU-10 scFvSA radioactivity in blood and most well-perfused soft tissues was very low, due to the low blood pool concentration induced by the sCA complexation and subsequent hepatic clearance. The exceptions were liver and tumor. Liver uptake and retention of fusion protein was due 15 to the mechanism of clearing agent action, and the somewhat retarded degradation of the streptavidin-containing fusion protein, which was consistent with similar results observed in studies of both streptavidin and the chemical conjugate of huNR-LU-10 and streptavidin (huNR-LU-10/SA) (data not shown). The <sup>125</sup>I-huNR-LU-10 scFvSA exhibited evidence of *in vivo* immunoreactivity by the retention of relatively high 20 radiolabel concentration at the tumor (both stoichiometrically and relative to blood pool concentration) at all time points. The ratio of tumor concentration to blood concentration continuously increased from 23 to 143 hours. The lower blood pool values induced by clearing agent have led to a dramatic increase in the ratio, achieving average values over twice those observed in the absence of clearing agent (data not 25 shown).

The pretargeted <sup>111</sup>In-DOTA-biotin biodistribution is shown in Figure 16. Consistent with pretargeting results employing the chemical conjugate huNR-LU-10/SA, the concentration of <sup>111</sup>In-DOTA-biotin radioactivity in blood and all non-xenograft soft tissues was very low. Despite the high concentrations of fusion 30 protein in the liver noted above, <sup>111</sup>In-DOTA-biotin uptake and retention in this organ was not evident, indicating that the fusion protein had been efficiently internalized and

was unavailable to bind the subsequently administered radiobiotin. The highest concentration of  $^{111}\text{In}$ -DOTA-biotin was at the tumor at all time points. (The tissues in order in Figure 16 are blood, tail, lung, liver, spleen, stomach, kidney, intestine, and tumor.) The rapid uptake, achieving peak concentrations at the earliest time point 5 sampled, is a hallmark of pretargeting. Efficient, consistent delivery and retention of  $^{111}\text{In}$ -DOTA-biotin at the tumor was also observed. Peak concentrations of  $^{111}\text{In}$ -DOTA-biotin at the tumor were within the range consistently achieved by use of the chemical NR-LU-10/SA conjugate (20-25 % injected dose/g) (data not shown).

10

#### EXAMPLE IX

##### ANALYSIS OF BLOOD CLEARANCE AND TUMOR UPTAKE OF HUNR-LU-10 SCFvSA VERSUS HUNR-LU-10/STREPTAVIDIN CHEMICAL CONJUGATE

15 Tumor to blood ratios of huNR-LU-10 scFvSA increased from nearly 100, two hours after DOTA-biotin injection, to several thousand by 24 hours. Comparative results for the huNR-LU-10/SA chemical conjugate and fusion protein, showing the efficiency of radiobiotin delivery to tumor and corresponding area-under-the-curve (AUC) values for blood, and tumor are shown in Figure 17.

20 The overall tumor AUC using the fusion protein was somewhat less than that of the chemical conjugate (1726, for the time interval between 0-120 hours, versus 2047 for a typical chemical conjugate experiment). However, there was a dramatic difference in the concentration of  $^{111}\text{In}$ -DOTA-biotin in the blood pool, with the concentration in the fusion protein group consistently lower at all time points. The 25 greatest ramification of this decreased retention of radioactivity in the blood is that animals treated with the fusion protein experience a higher therapeutic index (tumor/blood) than those treated with the chemical conjugate.

## EXAMPLE X

## PRETARGETED BIODISTRIBUTION OF B9E9 scFvSA

Pretargeted radioimmunotherapy studies were conducted in female nude mice bearing well-established Ramos human cancer xenografts (100-400 mg). Tumored Bkl:BALB/c/nu/nu nude mice were obtained by implanting 5-25 x 10<sup>6</sup> cultured cells subcutaneously in the side midline 10-25 days prior to study initiation. Mice received intravenous injections of the <sup>125</sup>I-labeled B9E9 scFvSA (600 µg), and 20 hours later were injected intravenously with 100 µg of synthetic clearing agent. <sup>111</sup>In-labeled DOTA-biotin (1.0 µg) was injected intravenously into each mouse 4 hours after clearing agent. Groups of three mice per time point were bled and sacrificed at 2, 24, and 48 hours after injection of <sup>111</sup>In-DOTA-biotin. Whole organs and tissue were isolated, weighed, and counted for radioactivity using a gamma counter.

As shown in Figure 18, the <sup>111</sup>In-DOTA-biotin radioactivity in blood and all non-xenograft soft tissues was below 2% of the injected dose/g. Further, <sup>111</sup>In-DOTA-biotin uptake and retention in liver is not seen, indicating that the fusion protein has been efficiently internalized by the liver, via the added clearing agent, and is unavailable to bind the subsequently administered radiobiotin. Stable delivery and retention of <sup>111</sup>In-DOTA-biotin at the tumor were observed. The highest concentration of radiobiotin at all time points was at the tumor (both stoichiometrically and relative to blood pool concentration). Peak concentrations of <sup>111</sup>In-DOTA-biotin at the tumor were 17-24 % of injected dose/g (mean 21.66, s.d. 3.17). Tumor to blood ratios increased from about 90, 2 hours after DOTA-biotin injection, to greater than 700 by 24 hours. In these experiments no effort was made to optimize the dose of the fusion protein, clearing agent, or DOTA-biotin, nor was any effort made to optimize the schedule of administration of these components. (In Figure 18, the tissues in order are blood, tail, lung, liver, spleen, stomach, kidney, intestine, and tumor.)

## CLAIMS

1. A vector construct for the expression of streptavidin fusion proteins, comprising:

5 (a) a first nucleic acid sequence encoding genomic streptavidin or a functional variant thereof;

(b) a promoter operatively linked to the first nucleic acid sequence; and

(c) a cloning site for insertion of a second nucleic acid sequence encoding a polypeptide to be fused with streptavidin, interposed between the promoter and the first nucleic acid sequence.

10 2. The construct of claim 1, wherein said construct further comprises said second nucleic acid sequence inserted at said cloning site.

3. The construct of claim 1, wherein the promoter is an inducible promoter.

4. The construct of claim 3, wherein the promoter is the Lac promoter.

5. The construct of claim 1, wherein the promoter is a constitutive promoter.

15 6. The construct of claim 1, further comprising *S. avidinii* regulatory sequences interposed between the promoter and the cloning site.

7. The construct of claim 6, wherein the regulatory sequence is a streptavidin regulatory sequence.

20 8. The construct of claim 1, further comprising a bacterial leader sequence interposed between the regulatory sequences and the cloning site.

9. The construct of claim 8, wherein the leader sequence comprises a signal sequence.

10. The construct of claim 8, wherein the leader sequence comprises a *S. avidinii* streptavidin signal sequence.

5 11. The construct of claim 10, wherein the signal sequence comprises nucleotides 50 to 121 of Figure 4.

12. The construct of claim 1, further comprising a nucleic acid sequence that encodes a protein that is a selectable marker.

10 13. The construct of claim 12, wherein the protein confers antibiotic resistance.

14. The construct of claim 1, wherein the first nucleic acid sequence encodes at least amino acids 14 to 150 of streptavidin, Figure 4.

15 15. The construct of claim 1, wherein the first nucleic acid sequence encodes at least amino acids selected from the group consisting of 1 to 158, 5 to 158, 14 to 150, 14 to 151, 14 to 152, 14 to 153, 14 to 154, 14 to 155, 14 to 156, 14 to 157, or 14 to 158 of streptavidin, Figure 4.

16. A host cell transfected with the construct of claim 1.

17. The host cell of claim 16, wherein the cell is selected from the group consisting of a bacterium, an insect, a plant, and a mammalian cell.

18. A fusion protein, comprising at least a first and a second polypeptide joined end to end, wherein said first polypeptide comprises at least 129 amino acids of streptavidin, Figure 4, or functional variants thereof, and wherein said second polypeptide comprises an amino acid sequence differing by at least one residue from said first polypeptide.

5 19. The fusion protein of claim 18, wherein said first and second polypeptides are separated by a linker of at least two amino acids.

20. The fusion protein of claim 19, wherein the linker is at least four amino acids.

10 21. The fusion protein of claim 20, wherein the linker is between four, five, six, seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, and twenty amino acids.

22. The fusion protein of claim 21, wherein the linker is between five to ten amino acids.

15 23. The fusion protein of claim 18, wherein said second polypeptide is an antibody or a fragment thereof.

24. The fusion protein of claim 23, wherein said fusion protein is capable of forming a tetrameric complex with a second, third, and fourth fusion protein, said second, third, and fourth fusion protein comprising at least a first and a second polypeptide joined end to end, wherein said first polypeptide comprises at least 129 amino acids of streptavidin, Figure 4, or functional variants thereof, and wherein said second polypeptide comprises an amino acid sequence differing by at least one residue from said first polypeptide.

25. The fusion protein of claim 23, wherein the antibody is B9E9.

26. The fusion protein of claim 23, wherein the antibody is a single-chain Fv fragment (scFv).

27. The fusion protein of claim 26, wherein the single-chain Fv fragment is derived from antibody B9E9.

28. The fusion protein of claim 26, wherein a linker connects the variable light and variable heavy chains of the single chain antibody.

29. The fusion protein of claim 28, wherein the linker comprises at least ten amino acid residues.

30. The fusion protein of claim 29, wherein the linker comprises at least fifteen amino acids.

31. The fusion protein of claim 30, wherein the linker comprises at least twenty amino acids.

32. The fusion protein of claim 31, wherein the linker comprises at least four Gly<sub>4</sub>Ser linkers.

33. The fusion protein of claim 23, wherein the antibody is specific for a cell surface protein or a cell-associated stromal or matrix protein.

34. The fusion protein of claim 33, wherein the antibody is a primatized antibody.

35. The fusion protein of claim 33, wherein the antibody is a murine antibody.

36. The fusion protein of claim 33, wherein the cell surface protein or the cell-associated stromal or matrix protein is selected from the group consisting of CD20, CD45, EGP40, CEA, TAG72, NCAM,  $\beta$ -HCG, a mucin, and neoangiogenic antigens.

5 37. The fusion protein of claim 36, wherein the cell surface protein is CD20.

38. The fusion protein of claim 18, wherein said first polypeptide comprises at least amino acids 14 to 150 of streptavidin, Figure 4.

10 39. The fusion protein of claim 18, wherein the first polypeptide comprises at least amino acids selected from the group consisting of 1 to 158, 5 to 158, 14 to 150, 14 to 151, 14 to 152, 14 to 153, 14 to 154, 14 to 155, 14 to 156, 14 to 157, or 14 to 158 of streptavidin, Figure 4.

15 40. A method for targeting a tumor cell comprising the administration of a fusion protein, said fusion protein comprising at least a first and a second polypeptide joined end to end, wherein said first polypeptide comprises at least 129 amino acids of streptavidin Figure 4, or conservatively substituted variants thereof, wherein said second polypeptide is a polypeptide which binds a cell surface protein, or a cell-associated stromal or matrix protein, on a tumor cell, wherein the fusion protein binds the cell surface protein, or a cell-associated stromal or matrix protein, on a tumor cell and wherein the streptavidin portion of the fusion protein is capable of binding biotin.

20 41. The method of claim 40, wherein the fusion protein binds a cell surface protein receptor, or a cell-associated stromal or matrix protein, on a tumor cell and a biotinylated radionuclide containing compound.

42. The method of claim 40, wherein said first and second polypeptides are separated by a linker of at least two amino acids.

43. The method of claim 42, wherein the linker is at least four amino acids.

44. The method of claim 43, wherein the linker is between four, five, six, 5 seven, eight, nine, ten, eleven, twelve, thirteen, fourteen, fifteen, sixteen, seventeen, eighteen, nineteen, and twenty amino acids.

45. The method of claim 44, wherein the linker is five to ten amino acids.

46. The method of claim 40, wherein the second polypeptide is an antibody.

47. The method of claim 46, wherein the antibody is B9E9.

10 48. The method of claim 40, wherein the antibody is a single-chain Fv fragment (scFv).

49. The method of claim 48, wherein the single-chain Fv fragment is derived from antibody B9E9.

15 50. The method of claim 48, wherein a linker connects the variable light and variable heavy chains of the single-chain antibody.

51. The method of claim 50, wherein the linker comprises at least ten amino acid residues.

52. The method of claim 51, wherein the linker comprises at least fifteen amino acids.

53. The method of claim 52, wherein the linker comprises at least twenty amino acids.

5 54. The method of claim 53, wherein the linker comprises at least four Gly<sub>4</sub>Ser linkers.

55. The method of claim 40, wherein the antibody specifically binds to a cell surface protein, or a cell-associated stromal or matrix protein, selected from the group consisting of CD20, CD22, CD45, CD52, CD56, CD57, EGP40, CEA, TAG-72, NCAM, -HCG, amucin, 10 EGF receptor, IL-2 receptor, her2/neu, Lewis y, GD2, GM2, tenascin, sialylated tenascin, somatostatin, activated tumor stromal antigen, and neoangiogenic antigens.

56. The method of claim 40, wherein the antibody is a primatized antibody.

57. The method of claim 40, wherein the antibody is a mouse antibody.

58. The method of claim 40, wherein said first polypeptide comprises at least 15 amino acids 14 to 158 of streptavidin Figure 4.

59. The method of claim 58, wherein said first polypeptide comprises at least amino acids 5 to 158 of streptavidin, Figure 4.

60. The method of claim 59, wherein said first polypeptide comprises at least amino acids 1 to 158 of streptavidin, Figure 4.

61. The method of claim 40, wherein the tumor cell is associated with a cancer selected from the group consisting of adenocarcinomas and hematological malignancies.

62. The method of claim 61, wherein the adenocarcinoma is selected from the group consisting of gliomas, prostate, ovarian, breast, colon, rectal, pancreatic, and lung.

5 63. The method of claim 61, wherein the hematological malignancy is selected from the group consisting of non-Hodgkin's lymphoma, Hodgkin's lymphoma, T-cell lymphoma, acute lymphocytic leukemia, acute myelogenous leukemia, chronic lymphocytic leukemia, chronic myelogenous leukemia, multiple myeloma, and Waldenstrom's macroglobulinemia.

10 64. A method for constructing a tetravalent antibody, comprising contacting the fusion protein of claim 23, wherein said fusion protein is capable of forming a tetrameric complex, with a second, third, and fourth fusion protein, said second, third, and fourth fusion protein also capable of forming a tetrameric complex and comprising at least a first and a second polypeptide joined end to end, wherein said first polypeptide comprises at least 129 amino acids of streptavidin, Figure 4, or functional variants thereof, and wherein said second polypeptide comprises an amino acid sequence differing by at least one residue from said first polypeptide.

15 65. A pharmaceutical composition, comprising the fusion protein of any one of claims 18-39.

66. A vector construct for the expression of streptavidin fusion proteins, comprising:

20 (a) a first nucleic acid sequence encoding a polypeptide to be fused with streptavidin;

(b) a promoter operatively linked to the first nucleic acid sequence; and

(c) a cloning site for insertion of a second nucleic acid sequence encoding at least 129 amino acids of streptavidin, Figure 4 or a functional variant thereof.

STREPTAVIDIN EXPRESSED GENE FUSIONS AND METHODS OF USE THEREOF

## ABSTRACT OF THE DISCLOSURE

5

The present invention provides vectors for expressing genomic streptavidin fusion cassettes. In the various embodiments, fusion proteins produced from these vectors are provided. In particular embodiments, fusion proteins comprising a single chain antibody and genomic streptavidin are provided as are vectors encoding the same. Also provided, are methods of using the fusion proteins of the present invention, and in particular, the use of scFvSA fusion proteins as diagnostic markers or as a cell specific targeting agents.

10

15

WPN/690022/547-AP/final



Figure 1

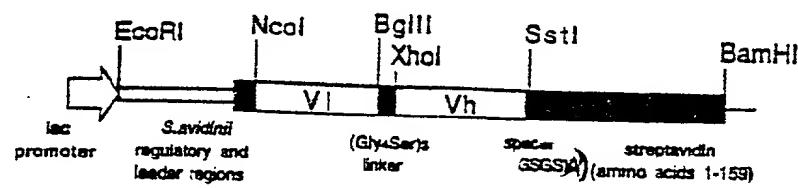


Figure 2

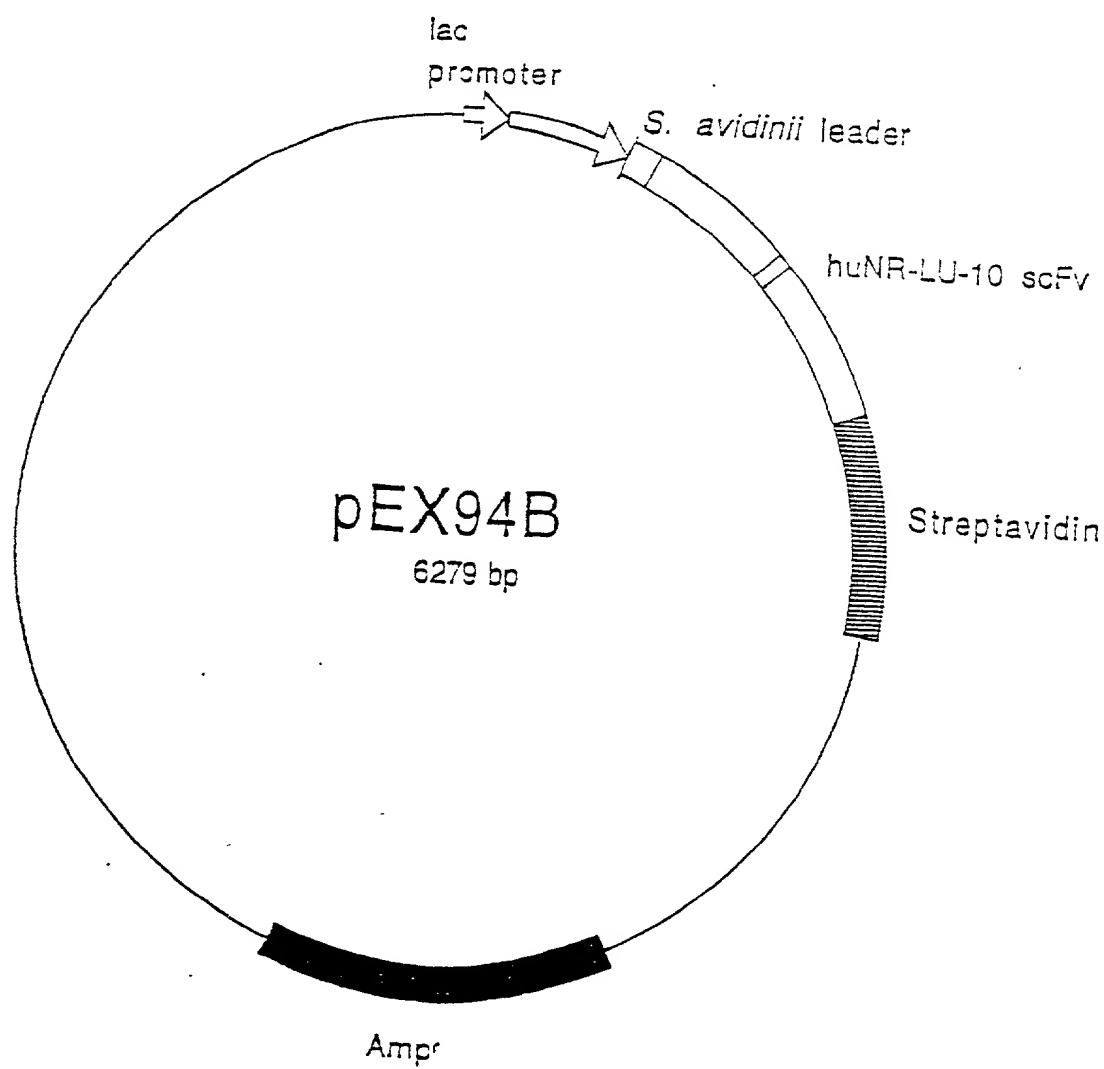


Figure 3

1 5' CCCCTCCGTCCCCCCCCGGGAAACAACTACGGAGATTTTCTGTCTCAC

-10

-20

-30

-40

-50

-60

-70

-80

-90

-100

-110

-120

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-160

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-180

-190

-200

-210

-220

-230

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-510

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-950

-960

-970

-980

-990

-1000

-1010

-1020

-1030

-1040

-1050

-1060

-1070

-1080

-1090

-1100

-1110

-1120

-1130

-1140

-1150

-1160

-1170

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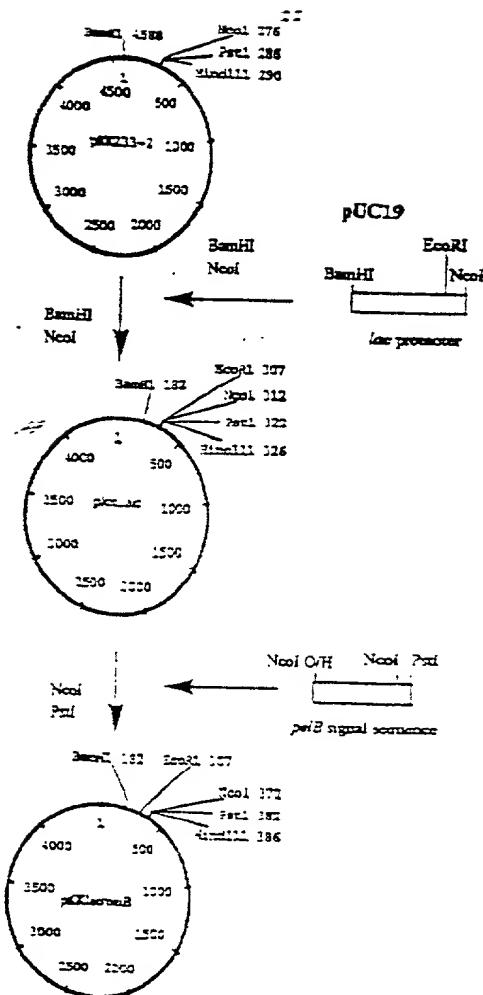


Figure 5

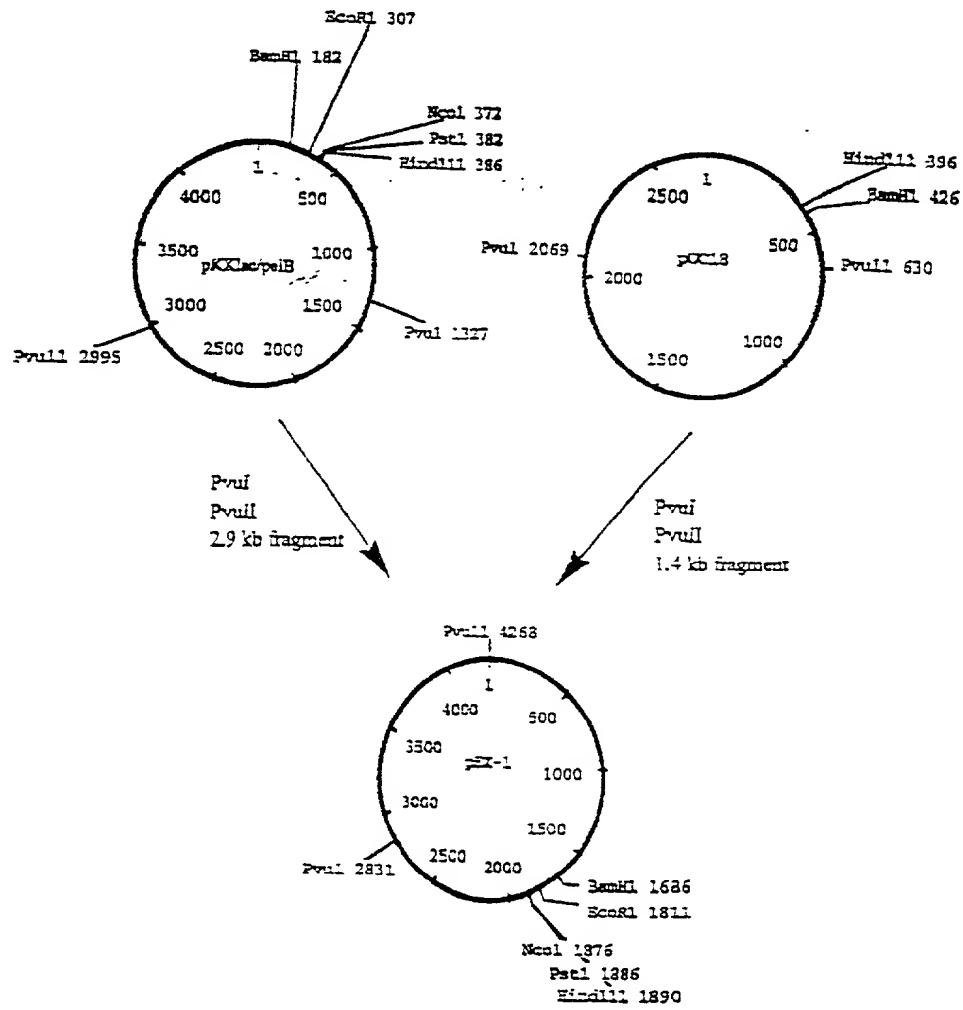


Figure 6

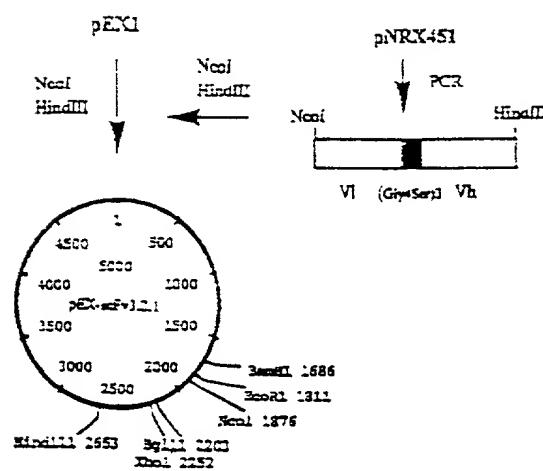
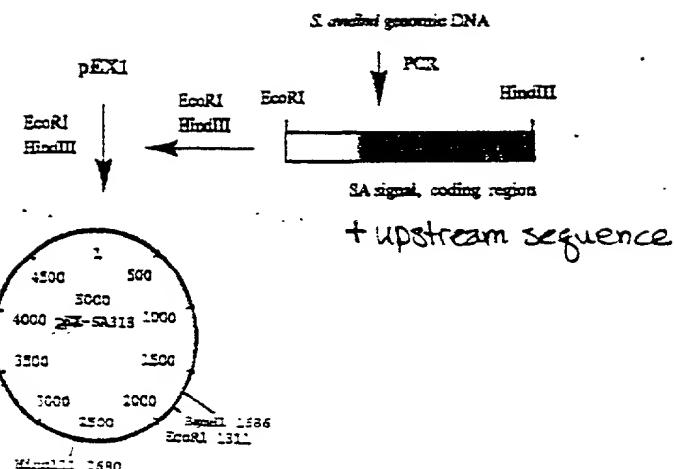


Figure 7

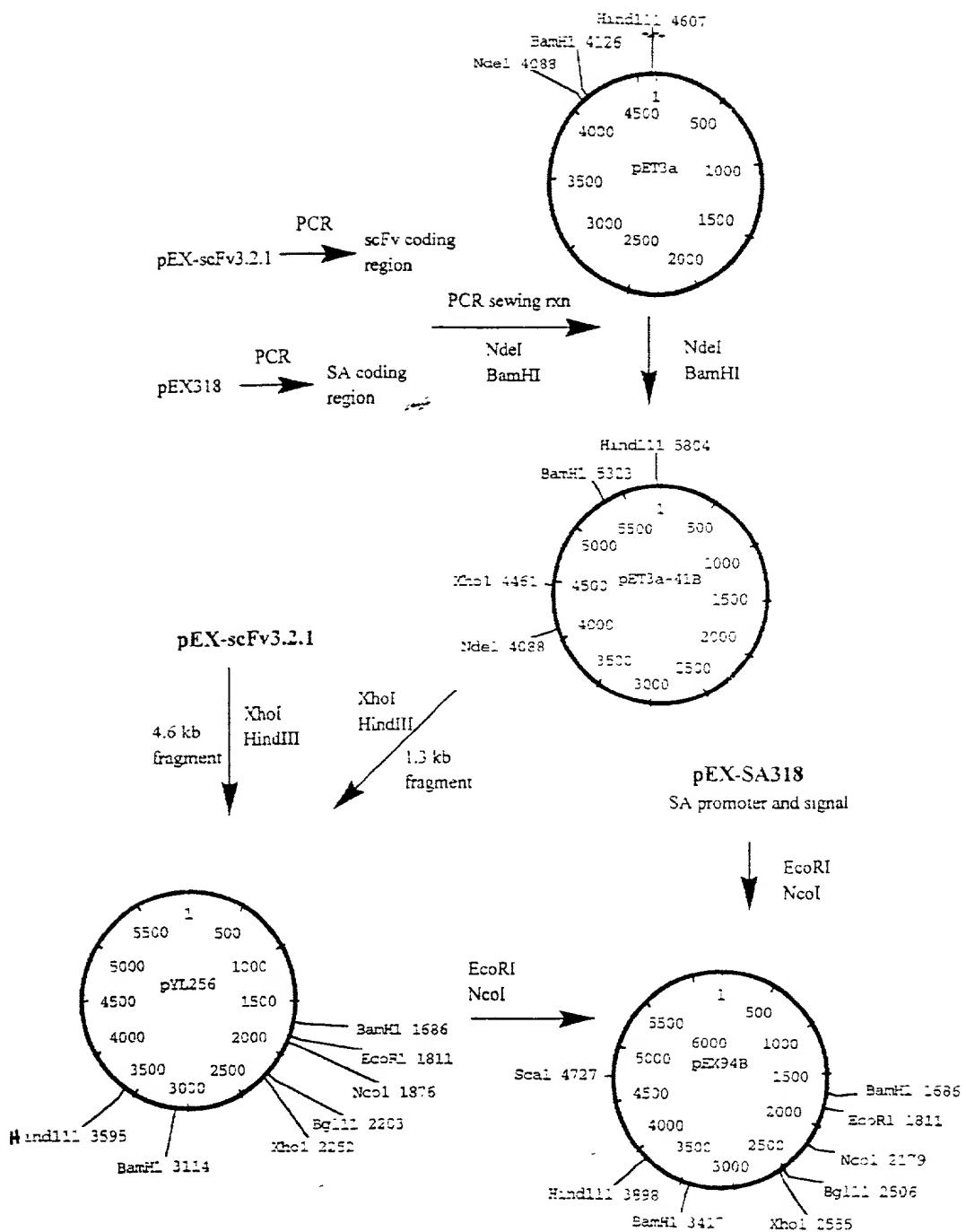


Figure 8

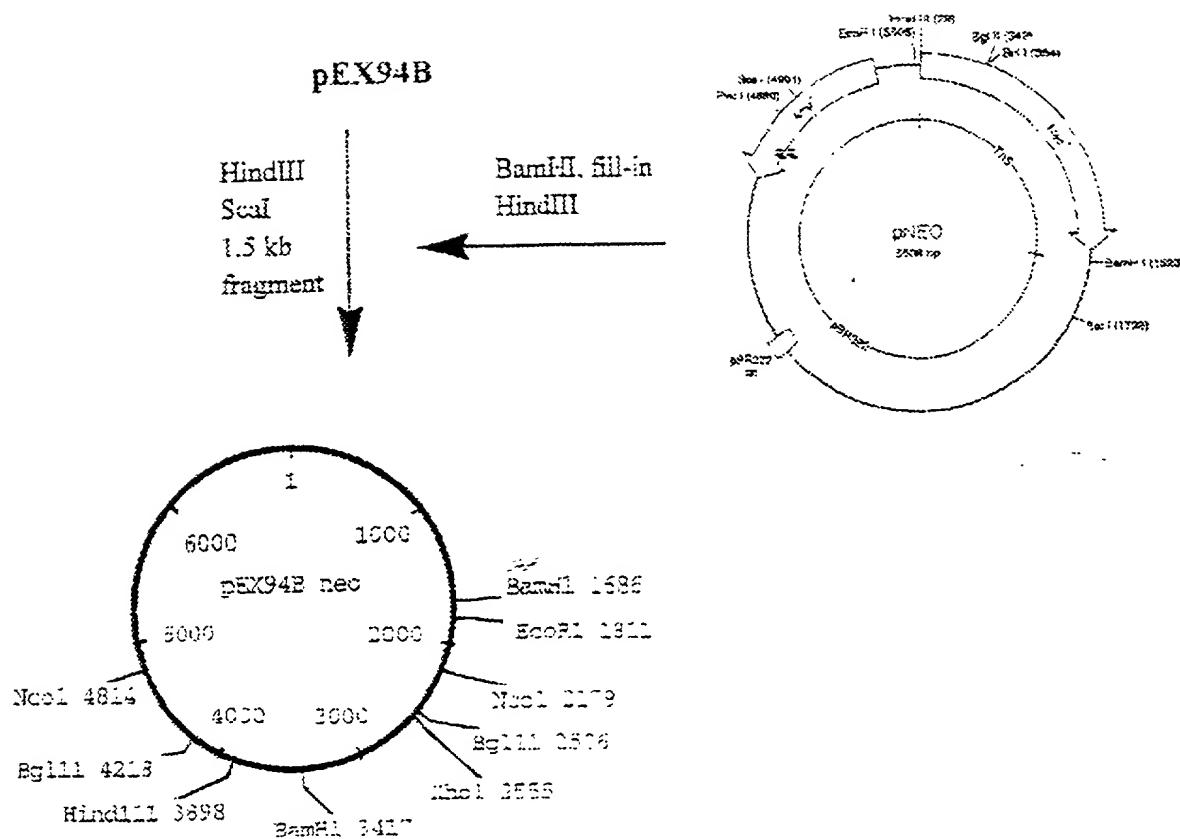


Figure 9

Figure 10

PEX94B EcoRI-BamHI fragment

1 GAATTCAAGGAACTTACCGACAGGACTCGGCGATCTTGGCGAAATTCCCTTGCAAGAAA  
 61 ATGTTGTTGAGAACCTCTGGATGGCTAGTACGATTCACCGAACATGTGCCCCCTGGCAA  
 121 CCACCGACCCGGACCTCGACCATCCAGTTCTGCCCGAACACACATGCCGACTGCTGT  
 181 TTGTTCAACCGAACACCTCAAGTGCACGGCGAGGTCAACACCTTGACGGGGGGATAAG  
 241 GACGGCGCAACCCACAGGGCGCCCTCGTCCCGCGGGCAACACTAGGGAGTATTTTT  
 301 CGTGTCTCACATGC3CAAGATCGTGTGCAACCGCTCGCGTTCTGACCAACGGTCTC  
 M R K I V V A A I A V S L T T V S  
 361 GATTAACGGCTTGGCTGACATCCAGACGACTCGTCTCCATCGTCCTGCTGCGCTGT  
 121 I T A M A D I Q M T Q S P S S L S A S V  
 signal peptidase  
 421 GGGAGACRGASTCACGATCCTTGTGGGCTAGTCAGGCAATTAGAGGTAAATTAGACTG  
 141 G D R V T I T C R A S Q G I R G N L D W  
 481 GTACAGCAGAAACCTGGTAAGGACCGAAACCTCATCTACTCCACATCCAAATTAAA  
 151 Y Q Q K P G K G P K L L I Y S T S N L N  
 541 TTCTGGTGTCCCATCAGGTCTGTGGCACTGGCTGGGTCAAGTTATCTCTCACCAT  
 161 S G V F S R F S G S G S G S D Y T L T I  
 601 CAGCAGCTTCAGCCTAACAGTTGGCAGCTTACTGTCTACAGCTAAATGCGTATCC  
 201 S S L Q P E D F A T Y Y C L Q R N A Y P  
 661 GTACACCTTCGGACAGGGACCGAGCTGGAGACCAAGATCTCTGGGGGTGGCTGGG  
 221 Y T F G Q G T K I S I K I S G G G G S G  
 linker  
 721 CGGTGGTGGGTGGGTGGGTGGGTGGGTGGGTGGGTGGGTGGGTGGGTGGGTGGGTGG  
 241 G G G S G G G G S S Q V Q L V Q S G A E  
 781 GGTGAAAAAGCCAGGGGCTCACTCAAGGTGTCTGGCAAGGTCTGGCTTCACATTAA  
 261 V K K P G A S V K V S C K A S G P N I K  
 841 AGACACCTTATTCACCTGGTGGAGGCGGGCCTGGACAGGGCCTGCTGGATGGGAAG  
 281 D T Y M H W V R Q A P G Q G L Q W M G R  
 901 GATTGATCTGGCAACGGTAATACTAAATCCACCTGGCTTCAGGGCAGGGTGACTAT  
 301 I D P A N G N T K S D L S F Q G R V T I  
 961 AACAGCAGACACGTCCATCACACAGCTACATGGACCTAGCAGGCTCAAGGTCTGACGA  
 321 T A D T S I N T A Y M Z L S S L R S D D  
 1021 CACTGGCGTCTTACTGTTAGAGAGGTCTTACTGGACGTGTCTTGGACTACTG  
 341 T A V Y Y C S R E V L T G T W S L D Y W  
 1081 GGGTCAAGGAACCTTGTGACCGTGGCTCTGGCTCTGGCTGGCGACCCCTGGCAAGGA

Streptavidin  
regulatory  
region

Streptavidin  
Signal sequence

V<sub>L</sub>

V<sub>H</sub>

Figure 10 Cont.

Linker

361 G Q G T L V T V S S G S G S A D P S K D  
 1141 CTCGAAGGCCCTGGCTCGGCCGCCAGGCCGGCTACCGGCTTGGTACACAGCT  
 381 S K A Q V S A A E A G I T G T W Y N Q L }  
 1201 CGGCTCGACCTTCATCGTACCGGCCGCCACGGGCCCTGACCGGAACCTACCGAGTC  
 401 G S T F I V T A G A D G A L T G T Y E S }  
 1261 GGCCTCGGCAACCCGAGGCCCTACGCTTGACCGCTTACGACACGCCGCCGCC  
 421 A V G N A E S R Y V L T G R Y D S A P A }  
 1321 CACCGACGGCAGGCCACCCCTGGCTGGACCGCTGGAAAGAATTAATACCGCAA  
 441 T D G S G T A L G W T V A W K N N Y R N }  
 1381 CGCCACTCGGCCACCGCTGGACCGGCCCTGGCTGGACCGCCAGGGCAGGATCAA  
 461 A H S A T T W S G Q Y V G G A E A R I N }  
 1441 CACCGCTGGCTGGTACCTCCGGCAACCGGCCACCCCTGGAAAGTCCACGCTGGT  
 481 T Q W L L T S G T T S A N A W K S T L V }  
 1501 CGGCCACGGACACCTTCACCGAGGTGAGGCCCTGCCGCCCTCCATCGACGGCGAAGAA  
 501 G H D T F T K V K P S A A S I D A A K K }  
 1561 GGCCTGGCTCAACGCCACGGCAACCGGCCCTGGACCGCTTACCGAGTAAGGATCC  
 521 A G V N N G N P L D A V Q Q \*

Streptavidin  
1-157

GACATCGTGC TGTCGCAGTC TCCAGCAATC CTGTCGCAT CTCCAGGGGA GAA  
 GGTCAACAATG ACTTGCAGGG CCAGCTCAAG TGTAAAGTAC ATGCACTGGT ACCAGCAGAA  
 GCCAGGATCC TCCCCAAAC CCTGGATTAA TGCCACATCC AACCTGGCTT CTGGAGTCCC  
 TGCTCGCTTC AGTGGCAGTG GGCTGGAC CTCTTACTCT CTCACAATCA GCAGAGTGGA  
 GGCTGAAGAT GCTGCCACTT ATTACTGCCA GCAGTGGATT AGTAACCCAC CCACGTCGG  
 TGCTGGGACC AAGCTGGAGC TGAAGATCTC TGGCTGGAA GGCAGCCCCG AAGCAGGTCT  
GTCTCCGGAC GCAGGTCTCG GCTCGAGCA GGTTCAGCTG GTCAGTCAG GGGCTGAGCT  
 GGTGAAGCCT GGGGCCTCAG TGAAGATGTC CTGCAAGGCT TCTGGCTACA CATTACCAAG  
 TTACAATATC CACTGGTAA AGCAGACACC TGGACAGGGC CTGGAATGGG TTGGAGCTAT  
 TTATCCAGGA AATGGTATA CTTCCTACAA TCAGAAGTTC AAAGGCAGG CCACATTGAC  
 TGCAGACAAA TCCCTCAGCA CAGCCTACAT GCAGCTCAG AGCCTGACAT CTGAGGACTC  
 TGCCTCTATTACTGTGCAA GAGCGCAATT ACGACCTAAC TACTGGTACT TCGATGTC  
 GGGCGCAGGG ACCACGGTC CCGTGAGGCTC TGGCTGGT TCGGCAGACC CCTCCAAGGA  
 CTGCAAGGCC CAGGTCTCGG CCGCCGAGGC CGGCATCACC GGCACCTGGT ACAACCAGCT  
 CGGCTCGACC TTCATCGTGA CGCGGGCGC CGACGGCGCC CTGACCGGAA CCTACGAGTC  
 GGCGTCCGGC AACGCCGAGA GCGCTACGT CCTGACCGGT CGTACGACA GCGCCCCGGC  
 CACCGACGGC AGCGGCACCG CCCTCGTTG GACGGTGGCC TGGAAAGAATA ACTACCGCAA  
 CGCCCACCTCC GCGACCACGT GGAGCGGCCA GTACGTCGGC GGCGCCGAGG CGAGGATCAA  
 CACCCAGTGG CTGCTGACCT CGGGCACCAC CGAGGCCAAC GCCTGGAAGT CCACGCTGGT  
 CGGCCACGAC ACCITCACCA AGGTGAAGCC GTCCGCCGCC TCCATCGACG CGGCAGAGAA  
 GGCGGGCTC AACAAACGGCA ACCCGCTCGA CGCCGTTAG CAGTAA

Translation of B9E9pKOD scFvSA

DIVLSQSPAIL SASPGEKVTM TCRASSSVSY MHWYQQKPGS SPKPWYIATS NLASGVPARF  
 SGSGSGTYSYS LTISRVEAED AATYFCQQWI SNPPTFGAGT KLELKIS GLE GSPEAGLSPD  
AGSGSS SQVQL VQSGAELVKP GASVKMSCKA SGYFTTSYNM HWVKQTPGQG LEWIGAIYPG  
 NGDTSYNQKF KGKATLTADK SSSTAYMQLS SLTSEDSAVY YCARAQLRPN YWYFDVWGAG  
 ITVTVSSGSG SADPSKDSKA QVSAAEAGIT GTWYNQLGST FIVTAGADGA LTGTYESAVG  
 NAESRYVLTG RYDSAPATDG SGTALGWTVA WKNINYRNAHS ATTWSGQYVG GAEARINTQW  
 LLTSGTTEAN AWKSTLVGHF TFTKVKPSAA SIDAACKAGV NNGNPLDAVQ Q\*

11A

Figure 11B

VL  
 pKOD  
 VH  
 Linker 2  
 SA

Figure 11C

E31-2-20 plasmid: NcoI-BamHI fragment containing B9E9 Vn-Maker-VI-SA genes

1 CCATGGCTCAGGTTCAAGCTGGTCCAGTCAGGGCTGACCTGGTGAAGCCTGGGCTCAG  
 1 M A Q V Q L V Q S G A E L V K P G A S V  
 61 TGAAGATGTCTGCAAGGCTTCTGGCTACACATTACCAAGTTACAATATGCACTGGTAA  
 21 K M S C K A S G Y T F T S Y N M H W V K  
 121 AGCAGACACCTGGACAGGGCTGGAAATGGATGGAGCTATTATCCAGGAAATGGTATA  
 41 Q T P G Q G L E W I G A I Y P G N G D T  
 181 CTTCTAACATCAGAAGTTCAGAGGCAAGGCCACATTGACTGCAGACAAATCTCCAGCA  
 61 S Y N Q K F K G K A T L T A D K S S S T  
 241 CAGCCTACATGCAGCTCAGCAGCCTGACATCTGAGGACTCTGGTCTATTACTGTGCAA  
 81 A Y M Q L S S L T S E D S A V Y Y C A R  
 301 GAGCCTAACATCGACCTAACTACTGGTACTTCGATGCTGGGCGCAGGGACCACGGTCA  
 101 A Q L R P N Y W Y F D V W G A G T T V T  
 361 CCGTGACCAAGATCTCTGGGGCTGGCTCGGGCGGTGGTGGCTGGCGGGCT  
 121 V S K I S G G G G S G G G G S G G G G S  
 421 CGGGTGGTGGTGGCTGGCGGGCGGGCTCGAGCGACATCGTGTGCGAGTCCTCCAG  
 141 G G G G S G G G G S S D I V L S Q S P A  
 481 CAACTCTGTCTGCATCTCCAGGGAGAAGGTACAATGACTTGCAGGGCCAGCTCAAGTG  
 161 I L S A S P G E K V T M T C R A S S S V  
 541 TAAGTTACATGCACTGGTACCAAGCAGAAGCAGGATCTCCCCAAACCTGGATTATG  
 181 S Y M H W Y Q Q K P G S S P K P W I Y A  
 601 CCACATCCACCTGGCTCTGGAGTCCCTGCTCAGTGGCAGTGGCTGGGACCT  
 201 T S N L A S G V P A R F S G S G S G T S  
 661 CTTACTCTCTCACAAATCAGCAGAGTGGAGGCTGAAGATGCTGCCACTTATTACTGCCAGC  
 221 Y S L T I S R V E A E D A A T Y Y C Q Q  
 721 AGTGGATTACTAACCCACCCACGGTTCGGTGGCTGGACCAAGCTGGAGCTGAAGAGCTCTG  
 241 W I S N P P T F G A G T K L E L K S S G  
 781 GCTCTGGTTGGCGAGACCCCTCCAAAGGACTCGAAGGCCAGGTCTGGCCGGAGGGCG  
 261 S G S A D P S K D S K A Q V S A A E A G  
 841 GCATCACCGGGCACCTGGTACACCAAGCTCGGCTGACCTTCATCGTACCCGGGGCGCG  
 281 I T G T W Y N Q L G S T F I V T A G A D  
 901 ACGGCGCCCTGACCGGAACCTACGGAGTCGGCCCTCGGCAACGGCGAGAGCCGCTACGTCC  
 301 G A L T G T Y E S A V G N A E S R Y V L  
 961 TGACCGGTCGTTACGACAGCGCCCCCCCCACGGACGGCAGGGCACCGCCCTCGTTGGA  
 321 T G R Y D S A P A T D G S G T A L G W T

V<sub>H</sub>

V<sub>L</sub>

Streptavidin

1021 CGGTGGCCTGGAAGAATAACTACCGCAACGCCACTCCGCACCACCTGGAGCGGCCAGT  
341 V A W K N N Y R N A H S A T T W S G Q Y  
1081 ACGTCGGCGCGCGAGGCGAGGATCAACACCCAGTGGCTGCTGACCTCCGGCACCCG  
361 V G G A E A R I N T Q W L L T S G T T E  
1141 AGGCCAACGCTGGAAGTCCACGGCTGGCCACGACACCTTCACCAAGGTGAAGCCGT  
381 A N A W K S T L V G H D T F T K V K P S  
1201 CGCCCGCTCCATCGACGCCGGCAAGAAGGCCGGCTAACACGGCAACCGCTCGACG  
401 A A S I D A A K K A G V N N G N P L D A  
1261 CCGTTCACCAAGTAAGGATCC  
421 V Q Q \* G S

FIG. 11C CONTINUED

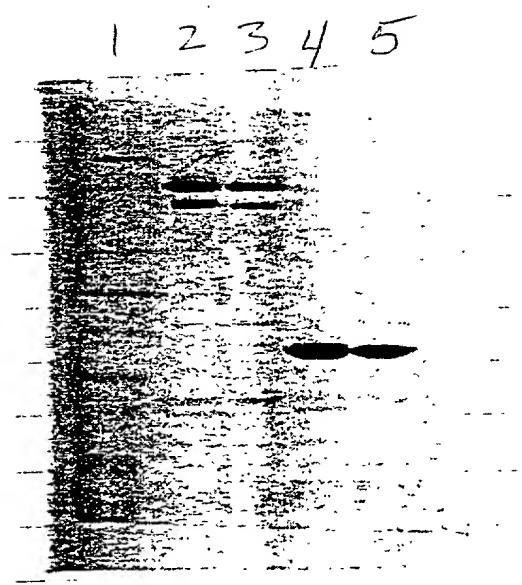


FIG. 12

### Size Exclusion HPLC

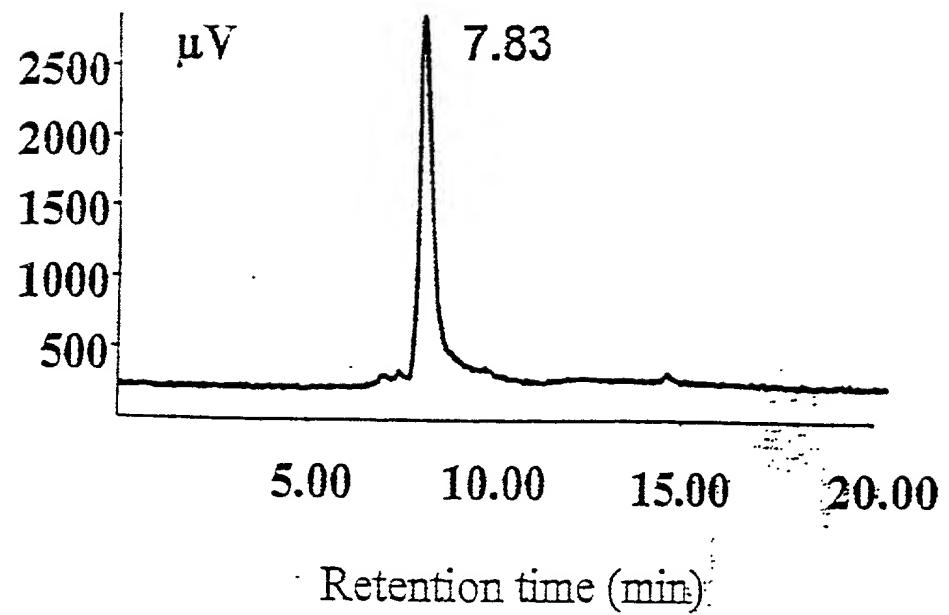


FIG. 13

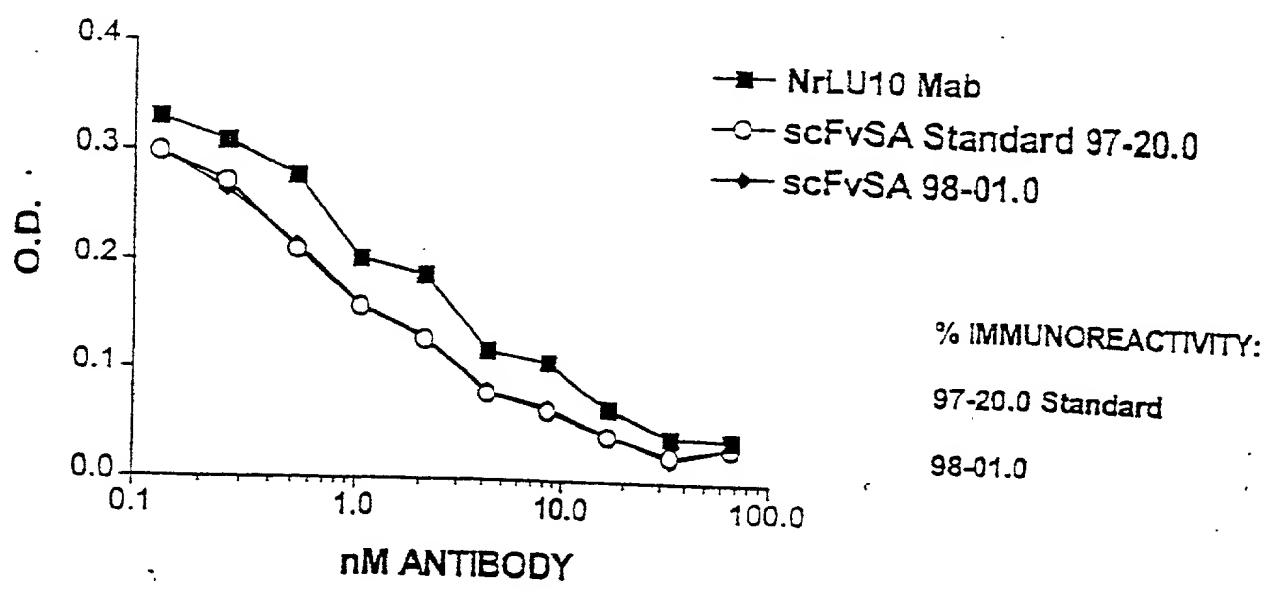


FIG. 14

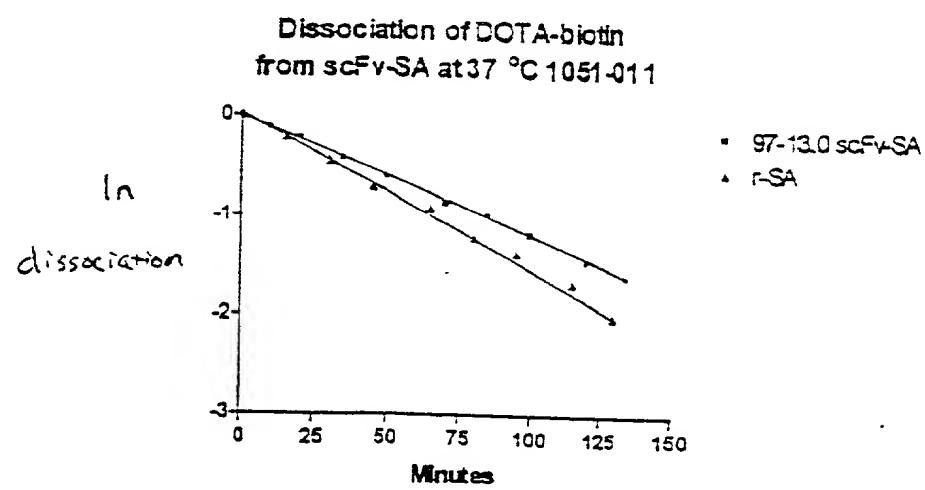


FIG. 15

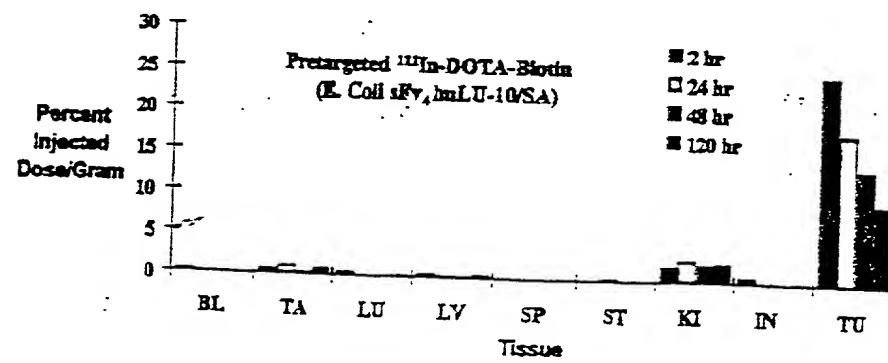


FIG. 16

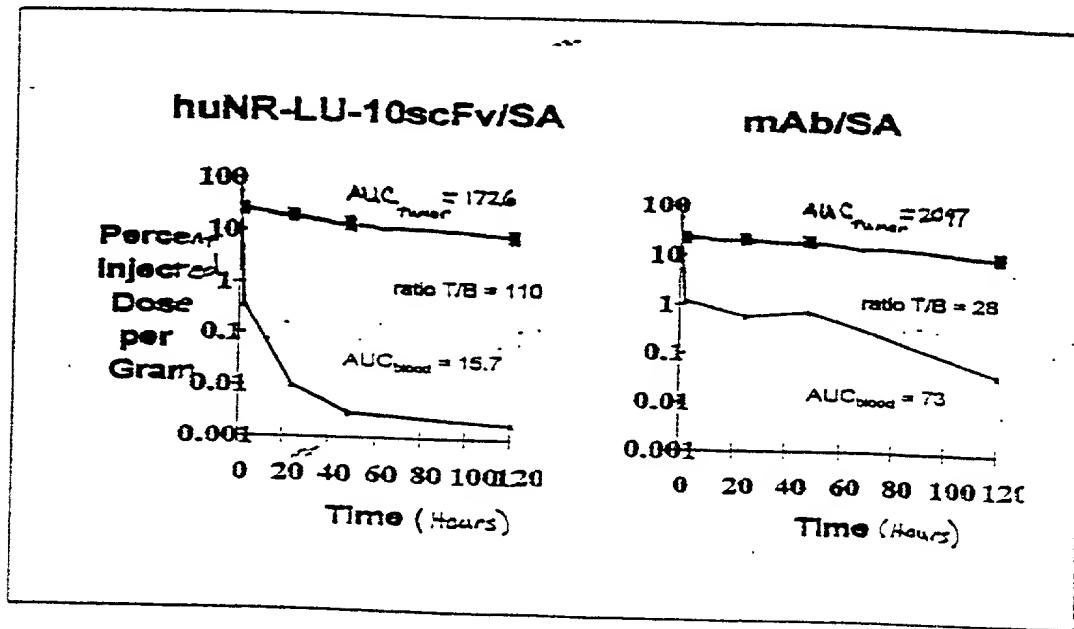


FIG. 17

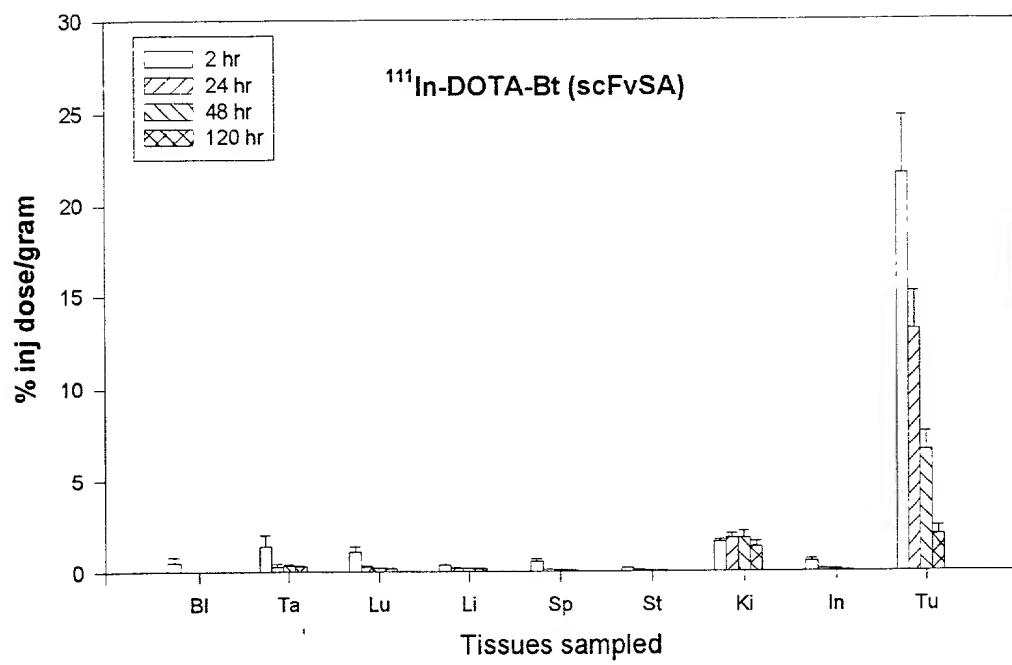


FIG. 18

Expression of scFvSA fusion proteins with or without FkpA.

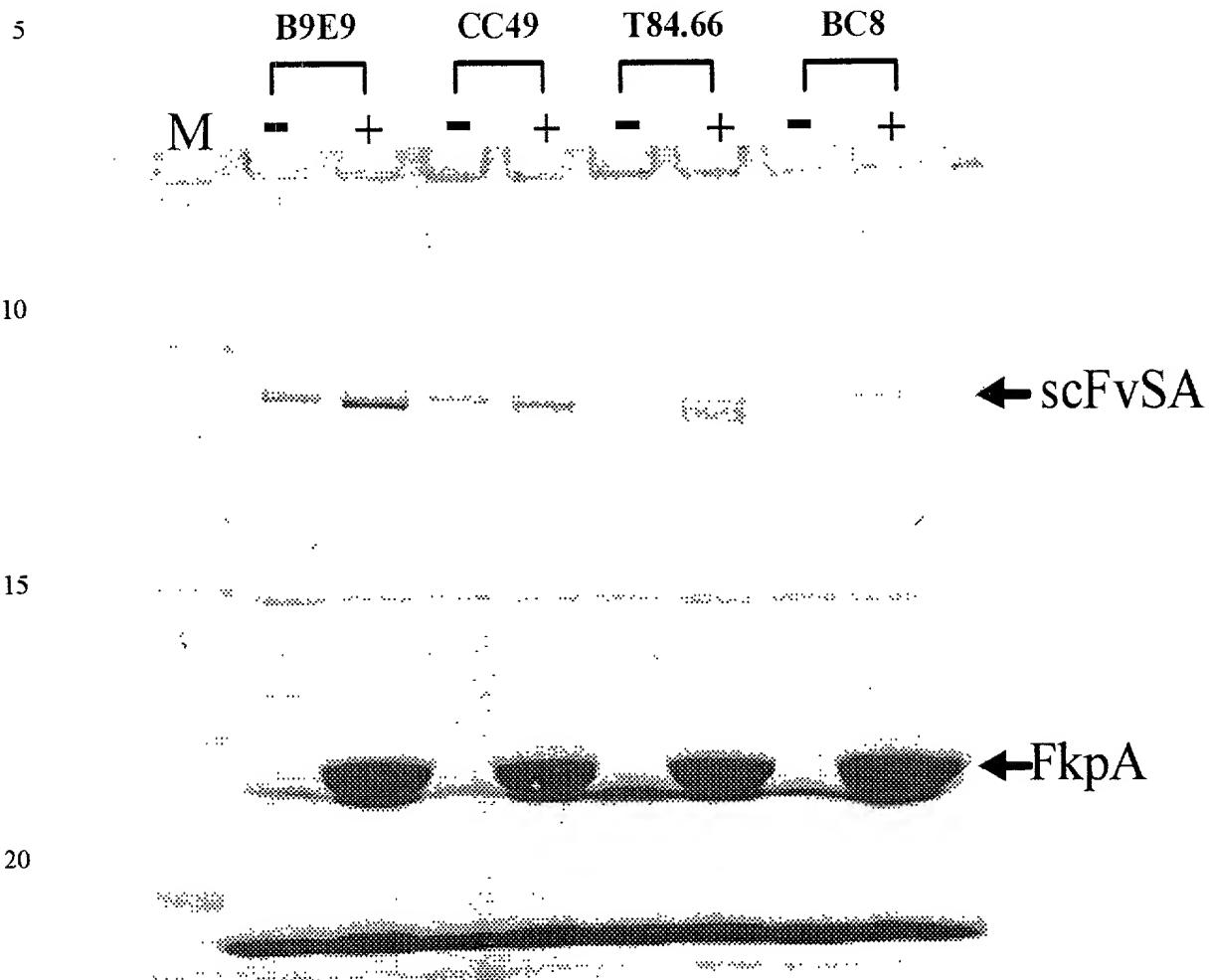


FIG. 19

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants : Stephen C. Goshorn, Scott Stoll Graves, Joanne Elaine Schultz,  
Yukang Lin, James A. Sanderson and John M. Reno

Filed : June 5, 2000

For : STREPTAVIDIN EXPRESSED GENE FUSIONS AND METHODS  
OF USE THEREOF

Docket No. : 690022.547

Date : June 5, 2000

Box Patent Application  
Assistant Commissioner for Patents  
Washington, D.C. 20231

DECLARATION

Sir:

I, Monica Steinborn, in accordance with 37 C.F.R. § 1.821(f) do hereby declare that, to the best of my knowledge, the content of the paper entitled "Sequence Listing" and the computer readable copy contained within the floppy disk are the same.

I declare further that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true and further that these statements are made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Dated this 5<sup>th</sup> day of June, 2000.

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| 50                                                              | 55  | 60  |
|-----------------------------------------------------------------|-----|-----|
| Gly Thr Tyr Glu Ser Ala Val Gly Asn Ala Glu Ser Arg Tyr Val Leu |     |     |
| 65                                                              | 70  | 75  |
| Thr Gly Arg Tyr Asp Ser Ala Pro Ala Thr Asp Gly Ser Gly Thr Ala |     |     |
| 85                                                              | 90  | 95  |
| Leu Gly Trp Thr Val Ala Trp Lys Asn Asn Tyr Arg Asn Ala His Ser |     |     |
| 100                                                             | 105 | 110 |
| Ala Thr Thr Trp Ser Gly Gln Tyr Val Gly Gly Ala Glu Ala Arg Ile |     |     |
| 115                                                             | 120 | 125 |
| Asn Thr Gln Trp Leu Leu Thr Ser Gly Thr Thr Glu Ala Asn Ala Trp |     |     |
| 130                                                             | 135 | 140 |
| Lys Ser Thr Leu Val Gly His Asp Thr Phe Thr Lys Val Lys Pro Ser |     |     |
| 145                                                             | 150 | 155 |
| Ala Ala Ser Ile Asp Ala Ala Lys Lys Ala Gly Val Asn Asn Gly Asn |     |     |
| 165                                                             | 170 | 175 |
| Pro Leu Asp Ala Val Gln Gln                                     |     |     |
| 180                                                             |     |     |

<210> 3  
 <211> 1612  
 <212> DNA  
 <213> Streptomyces avidinii

|                                                                     |  |      |
|---------------------------------------------------------------------|--|------|
| <400> 3                                                             |  |      |
| gaattcacga agtaaccgac aggactcggc cattcttgg ccgaaattcc tttgcagaaa    |  | 60   |
| atgttggta gaaccctccg atggcttagta cgatttacac cgaacatgtg cccttggcaa   |  | 120  |
| ccatcgaccc ggacctcgac catccagttc tgccgccaaa gacacatgcc gcactgctgt   |  | 180  |
| ttgttccaccc acaccgtcag gtgcacggcc gaggtcacaa accttgcacgg gcgggatacg |  | 240  |
| gacggcgcac gccacagcgc gccctccgtc cccggccggc aacaactagg gagtattttt   |  | 300  |
| cgtgtctcac atgcgcaaga tctgtcggtc agccatcgcc gtttccctga ccacggcttc   |  | 360  |
| gattacggcc atggctgaca tccagatgac tcagtttca tcgtcctgt ctgcctctgt     |  | 420  |
| gggagacaga gtcacgatca cttgtcggtc tagtcaggc attagaggtt atttagactg    |  | 480  |
| gtatcagcag aaacctggta agggaccgaa actcttatac tactccacat ccaattttaa   |  | 540  |
| ttctgggtgc ccatcaaggt tcagtggcag tgggtctggg tcagattata ctctcaccat   |  | 600  |
| cagcagcatt cagcctgaag atttcgcaac gtattactgt ctacagcgtt atgcgtatcc   |  | 660  |
| gtacacgttc ggacaaggga ccaagctgga gatcaagatc tctgggtggcg gtggctcggt  |  | 720  |
| cgggtgggg tcgggtggcg gaggctcgag ccaggttcag ctggtccagt ctggggcaga    |  | 780  |
| ggtaaaaaag ccaggggcct cagtcaaggt gtcctgcaag gtttctgct tcaacattaa    |  | 840  |
| agacacctat atgcactggg tgaggcaggc acctggacag ggcctgcagt ggatggaaag   |  | 900  |
| gattgatcct gcgaatggta atactaaatc cgacctgtcc ttccaggcga gggtaactat   |  | 960  |
| aacagcagac acgtccatca acacagccta catgaaactc agcagcctga ggtctgacga   |  | 1020 |
| cactgccgtc tattactgtt cttagagaggc cctaactggg acgtggtctt tggactactg  |  | 1080 |
| gggtcaaggg accttagtca ccgtgagcgc tggctctggt tcggcagacc cctccaaggaa  |  | 1140 |
| ctcgaaggcc caggctcggtt ccggccaggc cggcatcacc ggcacctggt acaaccagct  |  | 1200 |
| cggctcgacc ttcatcgta ccggggggcgc cgacggcgc ctgaccggaa cctacgagtc    |  | 1260 |
| ggccgtcgcc aacccggaga gcccgtacgt cctgaccggt cgttacgaca gcggccggc    |  | 1320 |
| caccgacggc agcggcaccg ccctcggtt gacgggtggcc tggaaagata actaccgcaa   |  | 1380 |
| cggccactcc gcgaccacgt ggagcggccca gtacgtcggtt ggcggccagg cgaggatcaa |  | 1440 |
| cacccagttt ctgtcgaccc cccgcaccac cgaggccaaac gcctggaaatg ccacgctgg  |  | 1500 |
| cggccacgac accttcacca aggtgaagcc gtccggcc tccatcgacg cggcgaagaa     |  | 1560 |
| ggccggcgcc aacaacggca acccgctcga cggcggttccag cagtaaggat cc         |  | 1612 |

<210> 4  
 <211> 431

&lt;212&gt; PRT

&lt;213&gt; Streptomyces avidinii

&lt;400&gt; 4

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Met | Arg | Lys | Ile | Val | Val | Ala | Ala | Ile | Ala | Val | Ser | Leu | Thr | Thr | Val |
| 1   |     |     |     | 5   |     |     |     |     | 10  |     |     |     | 15  |     |     |
| Ser | Ile | Thr | Ala | Met | Ala | Asp | Ile | Gln | Met | Thr | Gln | Ser | Pro | Ser | Ser |
|     |     |     |     |     |     |     | 20  |     | 25  |     |     |     | 30  |     |     |
| Leu | Ser | Ala | Ser | Val | Gly | Asp | Arg | Val | Thr | Ile | Thr | Cys | Arg | Ala | Ser |
|     |     |     |     |     | 35  |     |     | 40  |     |     | 45  |     |     |     |     |
| Gln | Gly | Ile | Arg | Gly | Asn | Leu | Asp | Trp | Tyr | Gln | Gln | Lys | Pro | Gly | Lys |
|     |     |     |     |     | 50  |     |     | 55  |     |     | 60  |     |     |     |     |
| Gly | Pro | Lys | Leu | Leu | Ile | Tyr | Ser | Thr | Ser | Asn | Leu | Asn | Ser | Gly | Val |
|     |     |     |     |     | 65  |     |     | 70  |     | 75  |     |     | 80  |     |     |
| Pro | Ser | Arg | Phe | Ser | Gly | Ser | Gly | Ser | Gly | Ser | Asp | Tyr | Thr | Leu | Thr |
|     |     |     |     |     | 85  |     |     | 90  |     |     | 95  |     |     |     |     |
| Ile | Ser | Ser | Leu | Gln | Pro | Glu | Asp | Phe | Ala | Thr | Tyr | Tyr | Cys | Leu | Gln |
|     |     |     |     |     | 100 |     |     | 105 |     |     | 110 |     |     |     |     |
| Arg | Asn | Ala | Tyr | Pro | Tyr | Thr | Phe | Gly | Gln | Gly | Thr | Lys | Leu | Glu | Ile |
|     |     |     |     |     | 115 |     |     | 120 |     |     | 125 |     |     |     |     |
| Lys | Ile | Ser | Gly | Gly | Gly | Ser | Gly | Gly | Gly | Gly | Ser | Gly | Gly | Gly | Gly |
|     |     |     |     |     | 130 |     |     | 135 |     |     | 140 |     |     |     |     |
| Gly | Ser | Ser | Gln | Val | Gln | Leu | Val | Gln | Ser | Gly | Ala | Glu | Val | Lys | Lys |
|     |     |     |     |     | 145 |     |     | 150 |     |     | 155 |     |     | 160 |     |
| Pro | Gly | Ala | Ser | Val | Lys | Val | Ser | Cys | Lys | Ala | Ser | Gly | Phe | Asn | Ile |
|     |     |     |     |     | 165 |     |     | 170 |     |     | 175 |     |     |     |     |
| Lys | Asp | Thr | Tyr | Met | His | Trp | Val | Arg | Gln | Ala | Pro | Gly | Gln | Gly | Leu |
|     |     |     |     |     | 180 |     |     | 185 |     |     | 190 |     |     |     |     |
| Gln | Trp | Met | Gly | Arg | Ile | Asp | Pro | Ala | Asn | Gly | Asn | Thr | Lys | Ser | Asp |
|     |     |     |     |     | 195 |     |     | 200 |     |     | 205 |     |     |     |     |
| Leu | Ser | Phe | Gln | Gly | Arg | Val | Thr | Ile | Thr | Ala | Asp | Thr | Ser | Ile | Asn |
|     |     |     |     |     | 210 |     |     | 215 |     |     | 220 |     |     |     |     |
| Thr | Ala | Tyr | Met | Glu | Leu | Ser | Ser | Leu | Arg | Ser | Asp | Asp | Thr | Ala | Val |
|     |     |     |     |     | 225 |     |     | 230 |     |     | 235 |     |     | 240 |     |
| Tyr | Tyr | Cys | Ser | Arg | Glu | Val | Leu | Thr | Gly | Thr | Trp | Ser | Leu | Asp | Tyr |
|     |     |     |     |     | 245 |     |     | 250 |     |     | 255 |     |     |     |     |
| Trp | Gly | Gln | Gly | Thr | Leu | Val | Thr | Val | Ser | Ser | Gly | Ser | Gly | Ser | Ala |
|     |     |     |     |     | 260 |     |     | 265 |     |     | 270 |     |     |     |     |
| Asp | Pro | Ser | Lys | Asp | Ser | Lys | Ala | Gln | Val | Ser | Ala | Ala | Glu | Ala | Gly |
|     |     |     |     |     | 275 |     |     | 280 |     |     | 285 |     |     |     |     |
| Ile | Thr | Gly | Thr | Trp | Tyr | Asn | Gln | Leu | Gly | Ser | Thr | Phe | Ile | Val | Thr |
|     |     |     |     |     | 290 |     |     | 295 |     |     | 300 |     |     |     |     |
| Ala | Gly | Ala | Asp | Gly | Ala | Leu | Thr | Gly | Thr | Tyr | Glu | Ser | Ala | Val | Gly |
|     |     |     |     |     | 305 |     |     | 310 |     |     | 315 |     |     | 320 |     |
| Asn | Ala | Glu | Ser | Arg | Tyr | Val | Leu | Thr | Gly | Arg | Tyr | Asp | Ser | Ala | Pro |
|     |     |     |     |     | 325 |     |     | 330 |     |     | 335 |     |     |     |     |
| Ala | Thr | Asp | Gly | Ser | Gly | Thr | Ala | Leu | Gly | Trp | Thr | Val | Ala | Trp | Lys |
|     |     |     |     |     | 340 |     |     | 345 |     |     | 350 |     |     |     |     |
| Asn | Asn | Tyr | Arg | Asn | Ala | His | Ser | Ala | Thr | Thr | Trp | Ser | Gly | Gln | Tyr |
|     |     |     |     |     | 355 |     |     | 360 |     |     | 365 |     |     |     |     |
| Val | Gly | Gly | Ala | Glu | Ala | Arg | Ile | Asn | Thr | Gln | Trp | Leu | Leu | Thr | Ser |
|     |     |     |     |     | 370 |     |     | 375 |     |     | 380 |     |     |     |     |
| Gly | Thr | Thr | Glu | Ala | Asn | Ala | Trp | Lys | Ser | Thr | Leu | Val | Gly | His | Asp |
|     |     |     |     |     | 385 |     |     | 390 |     |     | 395 |     |     | 400 |     |

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Thr | Phe | Thr | Lys | Val | Lys | Pro | Ser | Ala | Ala | Ser | Ile | Asp | Ala | Ala | Lys |
| 405 |     |     |     |     |     |     |     |     |     |     |     |     |     | 415 |     |
| Lys | Ala | Gly | Val | Asn | Asn | Gly | Asn | Pro | Leu | Asp | Ala | Val | Gln | Gln |     |
| 420 |     |     |     |     |     |     |     | 425 |     |     |     |     | 430 |     |     |

&lt;210&gt; 5

&lt;211&gt; 1239

&lt;212&gt; DNA

&lt;213&gt; Streptomyces avidinii

&lt;400&gt; 5

|             |             |             |             |            |             |      |
|-------------|-------------|-------------|-------------|------------|-------------|------|
| gacatcggtgc | tgtcgagtc   | tccagcaatc  | ctgtctgcat  | ctccaggggg | gaagggtcaca | 60   |
| atgacttgc   | gggcacgctc  | aagtgtaa    | gtacatgcact | ggtaccagca | gaagccagga  | 120  |
| tcctccccca  | aaccctggat  | ttatgccaca  | tccaacctgg  | cttctggagt | ccctgctcgc  | 180  |
| ttcagtggca  | gtgggtctgg  | gacctttac   | tctctcacaa  | tcagcagagt | ggaggctgaa  | 240  |
| gatgctgcca  | tttattactg  | ccagcagttgg | attagtaacc  | cacccacgtt | cggtgctggg  | 300  |
| accaagctgg  | agctgaagat  | ctctggcttg  | gaaggcagcc  | cggaagcagg | tctgtctccg  | 360  |
| gacgcagg    | ccggctcgag  | ccagggttcag | ctggtccagt  | caggggctga | gctgggtgaag | 420  |
| cctggggcct  | cagtgaagat  | gtcctgcaag  | gcttctggct  | acacatttac | cagttacaat  | 480  |
| atgcactggg  | taaagcagac  | acctggacag  | ggcctggaat  | ggattggagc | tatttatcca  | 540  |
| ggaaatgggt  | atacttccta  | caatcagaag  | ttcaaaaggca | aggccacatt | gactgcagac  | 600  |
| aaatcctcca  | gcacagccta  | catgcagctc  | agcagcctga  | catctgagga | ctctgcggtc  | 660  |
| tattactgtg  | caagagcgc   | attacgact   | aactactgg   | acttcgatgt | ctggggcgca  | 720  |
| gggaccacgg  | tcacogtgag  | ctctggctct  | ggttcggcag  | acccctccaa | ggactcgaag  | 780  |
| gcccgagg    | cgcccgccg   | ggccggcatc  | accggcacct  | ggtacaacca | gctcggtcg   | 840  |
| accttcatcg  | tgaccgcgg   | cgccgacg    | gcccgtaccg  | gaacctacga | gtcgccgtc   | 900  |
| ggcaacgccc  | agagccgcta  | cgtcctgacc  | ggtcgatcg   | acagcgcccc | ggccaccgac  | 960  |
| ggcagcggca  | ccgcctcgg   | ttggacggg   | gcttggaaaga | ataactaccg | caacgcccac  | 1020 |
| tccgcgacca  | cgtggagcgg  | ccagtgatcg  | ggcgccgccc  | aggcgaggat | caacacccag  | 1080 |
| tggctgtga   | cctccggcac  | caccgaggcc  | aacgcctgga  | agtccacgct | ggtcgccac   | 1140 |
| gacacccatca | ccaagggtgaa | gccgtccgac  | gcctccatcg  | acgcggcgaa | gaaggccggc  | 1200 |
| gtcaacaacg  | gcaacccgct  | cgacgcccgtt | cagcgtaa    |            |             | 1239 |

&lt;210&gt; 6

&lt;211&gt; 412

&lt;212&gt; PRT

&lt;213&gt; Streptomyces avidinii

&lt;400&gt; 6

|     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |     |
|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Asp | Ile | Val | Leu | Ser | Gln | Ser | Pro | Ala | Ile | Leu | Ser | Ala | Ser | Pro | Gly |
| 1   |     |     |     |     |     |     |     |     | 10  |     |     |     |     | 15  |     |
| Glu | Lys | Val | Thr | Met | Thr | Cys | Arg | Ala | Ser | Ser | Ser | Val | Ser | Tyr | Met |
|     |     |     |     |     |     |     |     |     | 20  | 25  |     |     |     | 30  |     |
| His | Trp | Tyr | Gln | Gln | Pro | Gly | Ser | Ser | Pro | Lys | Pro | Trp | Ile | Tyr |     |
|     |     |     |     |     |     |     |     |     | 35  | 40  |     |     |     | 45  |     |
| Ala | Thr | Ser | Asn | Leu | Ala | Ser | Gly | Val | Pro | Ala | Arg | Phe | Ser | Gly | Ser |
|     |     |     |     |     |     |     |     |     | 50  | 55  |     |     |     | 60  |     |
| Gly | Ser | Gly | Thr | Ser | Tyr | Ser | Leu | Thr | Ile | Ser | Arg | Val | Glu | Ala | Glu |
|     |     |     |     |     |     |     |     |     | 65  | 70  |     |     |     | 80  |     |
| Asp | Ala | Ala | Thr | Tyr | Tyr | Cys | Gln | Gln | Trp | Ile | Ser | Asn | Pro | Pro | Thr |
|     |     |     |     |     |     |     |     |     | 85  | 90  |     |     |     | 95  |     |
| Phe | Gly | Ala | Gly | Thr | Lys | Leu | Glu | Leu | Lys | Ile | Ser | Gly | Leu | Glu | Gly |
|     |     |     |     |     |     |     |     |     | 100 | 105 |     |     |     | 110 |     |
| Ser | Pro | Glu | Ala | Gly | Leu | Ser | Pro | Asp | Ala | Gly | Ser | Gly | Ser | Ser | Gln |

|                                                                 |     |     |
|-----------------------------------------------------------------|-----|-----|
| 115                                                             | 120 | 125 |
| Val Gln Leu Val Gln Ser Gly Ala Glu Leu Val Lys Pro Gly Ala Ser |     |     |
| 130                                                             | 135 | 140 |
| Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr Ser Tyr Asn |     |     |
| 145                                                             | 150 | 155 |
| Met His Trp Val Lys Gln Thr Pro Gly Gln Gly Leu Glu Trp Ile Gly |     |     |
| 165                                                             | 170 | 175 |
| Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln Lys Phe Lys |     |     |
| 180                                                             | 185 | 190 |
| Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr Ala Tyr Met |     |     |
| 195                                                             | 200 | 205 |
| Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr Tyr Cys Ala |     |     |
| 210                                                             | 215 | 220 |
| Arg Ala Gln Leu Arg Pro Asn Tyr Trp Tyr Phe Asp Val Trp Gly Ala |     |     |
| 225                                                             | 230 | 235 |
| Gly Thr Thr Val Thr Val Ser Ser Gly Ser Gly Ser Ala Asp Pro Ser |     |     |
| 245                                                             | 250 | 255 |
| Lys Asp Ser Lys Ala Gln Val Ser Ala Ala Glu Ala Gly Ile Thr Gly |     |     |
| 260                                                             | 265 | 270 |
| Thr Trp Tyr Asn Gln Leu Gly Ser Thr Phe Ile Val Thr Ala Gly Ala |     |     |
| 275                                                             | 280 | 285 |
| Asp Gly Ala Leu Thr Gly Thr Tyr Glu Ser Ala Val Gly Asn Ala Glu |     |     |
| 290                                                             | 295 | 300 |
| Ser Arg Tyr Val Leu Thr Gly Arg Tyr Asp Ser Ala Pro Ala Thr Asp |     |     |
| 305                                                             | 310 | 315 |
| Gly Ser Gly Thr Ala Leu Gly Trp Thr Val Ala Trp Lys Asn Asn Tyr |     |     |
| 325                                                             | 330 | 335 |
| Arg Asn Ala His Ser Ala Thr Thr Trp Ser Gly Gln Tyr Val Gly Gly |     |     |
| 340                                                             | 345 | 350 |
| Ala Glu Ala Arg Ile Asn Thr Gln Trp Leu Leu Thr Ser Gly Thr Thr |     |     |
| 355                                                             | 360 | 365 |
| Glu Ala Asn Ala Trp Lys Ser Thr Leu Val Gly His Asp Thr Phe Thr |     |     |
| 370                                                             | 375 | 380 |
| Lys Val Lys Pro Ser Ala Ala Ser Ile Asp Ala Ala Lys Lys Ala Gly |     |     |
| 385                                                             | 390 | 395 |
| Val Asn Asn Gly Asn Pro Leu Asp Ala Val Gln Gln                 |     |     |
| 405                                                             | 410 |     |

<210> 7  
 <211> 1280  
 <212> DNA  
 <213> Streptomyces avidinii

<400> 7

|                                                                    |     |
|--------------------------------------------------------------------|-----|
| ccatggctca ggttcagtcg gtccagtcag gggctgagct ggtgaaggct ggggcctcag  | 60  |
| tgaagatgtc ctgcaaggct tctggctaca catttaccag ttacaatatg cactggtaa   | 120 |
| agcagacacc tggacagggc ctgaaatgga ttggagctat ttatccagga aatgggtata  | 180 |
| cttcctacaa tcagaagttc aaaggcaagg ccacattgac tgcagacaaa tcctccagca  | 240 |
| cagcctacat gcagctcagc agcctgacat ctgaggactc tgcggcttat tactgtgcaa  | 300 |
| gagcgcatt acgacctaac tactggtaact tcgatgtctg gggcgcaggg accacggta   | 360 |
| ccgtgagcaa gatctctggt ggcggtggt cggggcggtgg tgggtcggtt ggccggcggt  | 420 |
| cgggtgggtgg tgggtcggtt ggcggcggtt cgagcgacat cgtgctgtcg cagtctccag | 480 |
| caatcctgtc tgcacatcttca ggggagaagg tcacaatgac ttgcagggcc agctcaagt | 540 |
| taagttacat gcactggta cagcagaagc caggatcctc ccccaaaccc tggatttatg   | 600 |

|                                                                     |      |
|---------------------------------------------------------------------|------|
| ccacatccaa cctggcttct ggagtccctg ctgcgttcag tggcagtggg tctggacat    | 660  |
| cttactctct cacaatcagc agagtggagg ctgaagatgc tgccacttat tactgccagc   | 720  |
| agtggattag taaccaccc acgttcgggtg ctgggaccaa gctggagctg aagagctctg   | 780  |
| gctctgggttc ggcagacccc tccaaggact cgaaggccca ggtctcgcc gccgaggccg   | 840  |
| gcatcaccgg cacctggtaa aaccagctcg gctcgacctt catcgtaacc gccccggccg   | 900  |
| acggcgccct gaccggaaacc tacgagtcgg ccgtcgccaa cggcggagagc cgctacgtcc | 960  |
| tgaccggctcg ttacgacagc gccccggcca ccgacggcag cggcaccggc ctcggttgg   | 1020 |
| cggtggcctg gaagaataac taccgcaacg cccactccgc gaccacgtgg agcggccagt   | 1080 |
| acgtcgccgg cggccggaggc aggatcaaca cccagtggct gctgacctcc ggcaccaccc  | 1140 |
| aggccaacgc ctggaaagtcc acgctggctg gccacgacac cttcaccaag gtgaagccgt  | 1200 |
| ccggccgcctc catcgacgcg gcgaaagaagg ccggcgtcaa caacggcaac ccgctcgacg | 1260 |
| ccggttccagca gtaaggatcc                                             | 1280 |

<210> 8  
<211> 423  
<212> PRT  
<213> Streptomyces avidinii

|                                                                 |  |
|-----------------------------------------------------------------|--|
| <400> 8                                                         |  |
| Met Ala Gln Val Gln Leu Val Gln Ser Gly Ala Glu Leu Val Lys Pro |  |
| 1 5 10 15                                                       |  |
| Gly Ala Ser Val Lys Met Ser Cys Lys Ala Ser Gly Tyr Thr Phe Thr |  |
| 20 25 30                                                        |  |
| Ser Tyr Asn Met His Trp Val Lys Gln Thr Pro Gly Gln Gly Leu Glu |  |
| 35 40 45                                                        |  |
| Trp Ile Gly Ala Ile Tyr Pro Gly Asn Gly Asp Thr Ser Tyr Asn Gln |  |
| 50 55 60                                                        |  |
| Lys Phe Lys Gly Lys Ala Thr Leu Thr Ala Asp Lys Ser Ser Ser Thr |  |
| 65 70 75 80                                                     |  |
| Ala Tyr Met Gln Leu Ser Ser Leu Thr Ser Glu Asp Ser Ala Val Tyr |  |
| 85 90 95                                                        |  |
| Tyr Cys Ala Arg Ala Gln Leu Arg Pro Asn Tyr Trp Tyr Phe Asp Val |  |
| 100 105 110                                                     |  |
| Trp Gly Ala Gly Thr Thr Val Thr Val Ser Lys Ile Ser Gly Gly Gly |  |
| 115 120 125                                                     |  |
| Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Gly Gly     |  |
| 130 135 140                                                     |  |
| Ser Gly Gly Gly Ser Ser Asp Ile Val Leu Ser Gln Ser Pro Ala     |  |
| 145 150 155 160                                                 |  |
| Ile Leu Ser Ala Ser Pro Gly Glu Lys Val Thr Met Thr Cys Arg Ala |  |
| 165 170 175                                                     |  |
| Ser Ser Ser Val Ser Tyr Met His Trp Tyr Gln Gln Lys Pro Gly Ser |  |
| 180 185 190                                                     |  |
| Ser Pro Lys Pro Trp Ile Tyr Ala Thr Ser Asn Leu Ala Ser Gly Val |  |
| 195 200 205                                                     |  |
| Pro Ala Arg Phe Ser Gly Ser Gly Ser Gly Thr Ser Tyr Ser Leu Thr |  |
| 210 215 220                                                     |  |
| Ile Ser Arg Val Glu Ala Glu Asp Ala Ala Thr Tyr Tyr Cys Gln Gln |  |
| 225 230 235 240                                                 |  |
| Trp Ile Ser Asn Pro Pro Thr Phe Gly Ala Gly Thr Lys Leu Glu Leu |  |
| 245 250 255                                                     |  |
| Lys Ser Ser Gly Ser Gly Ser Ala Asp Pro Ser Lys Asp Ser Lys Ala |  |
| 260 265 270                                                     |  |
| Gln Val Ser Ala Ala Glu Ala Gly Ile Thr Gly Thr Trp Tyr Asn Gln |  |

|                                 |                     |                     |
|---------------------------------|---------------------|---------------------|
| 275                             | 280                 | 285                 |
| Leu Gly Ser Thr Phe Ile Val     | Thr Ala Gly Ala Asp | Gly Ala Leu Thr     |
| 290                             | 295                 | 300                 |
| Gly Thr Tyr Glu Ser Ala Val     | Gly Asn Ala Glu Ser | Arg Tyr Val Leu     |
| 305                             | 310                 | 315                 |
| Thr Gly Arg Tyr Asp Ser Ala Pro | Ala Thr Asp Gly Ser | Gly Thr Ala         |
| 325                             | 330                 | 335                 |
| Leu Gly Trp Thr Val Ala Trp Lys | Asn Asn Tyr Arg Asn | Ala His Ser         |
| 340                             | 345                 | 350                 |
| Ala Thr Thr Trp Ser Gly Gln     | Tyr Val Gly Gly     | Ala Glu Ala Arg Ile |
| 355                             | 360                 | 365                 |
| Asn Thr Gln Trp Leu Leu Thr     | Ser Gly Thr Thr     | Glu Ala Asn Ala Trp |
| 370                             | 375                 | 380                 |
| Lys Ser Thr Leu Val Gly His Asp | Thr Phe Thr Lys Val | Lys Pro Ser         |
| 385                             | 390                 | 395                 |
| Ala Ala Ser Ile Asp Ala Ala Lys | Lys Ala Gly Val     | Asn Asn Gly Asn     |
| 405                             | 410                 | 415                 |
| Pro Leu Asp Ala Val Gln         |                     |                     |
| 420                             |                     |                     |

<210> 9  
 <211> 18  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> pKOD linker

<400> 9  
 Gly Leu Glu Gly Ser Pro Glu Ala Gly Leu Ser Pro Asp Ala Gly Ser  
 1 5 10 15  
 Gly Ser

<210> 10  
 <211> 15  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Linker used to create a scFvSA version of  
 anti-CD20mAb, B9E9 in the VLVH orientation

<400> 10  
 Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser  
 1 5 10 15

<210> 11  
 <211> 25  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Linker used to create a version of B9E9 scFvSA in

the VHVL orientation

<400> 11  
 Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly  
 1 5 10 15  
 Gly Gly Gly Ser Gly Gly Gly Ser  
 20 25

<210> 12  
 <211> 32  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Oligonucleotide primer

<400> 12  
 tgccgtgaat tcgttsmarct gcagsartcw gg

32

<210> 13  
 <211> 31  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Oligonucleotide primer

<400> 13  
 tgccgtgaat tccattswgc tgaccartct c

31

<210> 14  
 <211> 35  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Oligonucleotide primer

<400> 14  
 tagctggcgg ccgcctgtt gaagctcttg acaat

35

<210> 15  
 <211> 34  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Oligonucleotide primer

<400> 15  
 tagctggcgg ccgccttctt gtccaccttg gtgc

34

<210> 16  
 <211> 47

|                                                                  |  |    |
|------------------------------------------------------------------|--|----|
| <212> DNA                                                        |  |    |
| <213> Artificial Sequence                                        |  |    |
| <br>                                                             |  |    |
| <220>                                                            |  |    |
| <223> Oligonucleotide primer                                     |  |    |
| <br>                                                             |  |    |
| <400> 16                                                         |  |    |
| ttacggccat ggctgacatc gtgctgcagt ctccagcaat cctgtct              |  | 47 |
| <br>                                                             |  |    |
| <210> 17                                                         |  |    |
| <211> 32                                                         |  |    |
| <212> DNA                                                        |  |    |
| <213> Artificial Sequence                                        |  |    |
| <br>                                                             |  |    |
| <220>                                                            |  |    |
| <223> Oligonucleotide primer                                     |  |    |
| <br>                                                             |  |    |
| <400> 17                                                         |  |    |
| caccagagat cttcagctcc agcttggtcc ca                              |  | 32 |
| <br>                                                             |  |    |
| <210> 18                                                         |  |    |
| <211> 52                                                         |  |    |
| <212> DNA                                                        |  |    |
| <213> Artificial Sequence                                        |  |    |
| <br>                                                             |  |    |
| <220>                                                            |  |    |
| <223> Oligonucleotide primer                                     |  |    |
| <br>                                                             |  |    |
| <400> 18                                                         |  |    |
| cgaggaggctcg agccagggttc agctggtcca gtcaggggct gagctggtga ag     |  | 52 |
| <br>                                                             |  |    |
| <210> 19                                                         |  |    |
| <211> 38                                                         |  |    |
| <212> DNA                                                        |  |    |
| <213> Artificial Sequence                                        |  |    |
| <br>                                                             |  |    |
| <220>                                                            |  |    |
| <223> Oligonucleotide primer                                     |  |    |
| <br>                                                             |  |    |
| <400> 19                                                         |  |    |
| gagccagagc tcacggtgac cgtggccct gcgcccca                         |  | 38 |
| <br>                                                             |  |    |
| <210> 20                                                         |  |    |
| <211> 58                                                         |  |    |
| <212> DNA                                                        |  |    |
| <213> Artificial Sequence                                        |  |    |
| <br>                                                             |  |    |
| <220>                                                            |  |    |
| <223> Oligonucleotide primer                                     |  |    |
| <br>                                                             |  |    |
| <400> 20                                                         |  |    |
| gatctctggc ctggaaggca gcccggaaagc aggtctgtct ccggacgcag gttccggc |  | 58 |
| <br>                                                             |  |    |
| <210> 21                                                         |  |    |

|                                                                 |  |    |
|-----------------------------------------------------------------|--|----|
| <211> 58                                                        |  |    |
| <212> DNA                                                       |  |    |
| <213> Artificial Sequence                                       |  |    |
| <br>                                                            |  |    |
| <220>                                                           |  |    |
| <223> Oligonucleotide primer                                    |  |    |
| <br>                                                            |  |    |
| <400> 21                                                        |  |    |
| tcgagccgga acctgcgtcc ggagacagac ctgcttccgg gctgccttcc agaccaga |  | 58 |
| <br>                                                            |  |    |
| <210> 22                                                        |  |    |
| <211> 50                                                        |  |    |
| <212> DNA                                                       |  |    |
| <213> Artificial Sequence                                       |  |    |
| <br>                                                            |  |    |
| <220>                                                           |  |    |
| <223> Oligonucleotide primer                                    |  |    |
| <br>                                                            |  |    |
| <400> 22                                                        |  |    |
| ttacggccat ggctgacatc gtgctgtcgc agtctccagc aatcctgtct          |  | 50 |
| <br>                                                            |  |    |
| <210> 23                                                        |  |    |
| <211> 37                                                        |  |    |
| <212> DNA                                                       |  |    |
| <213> Artificial Sequence                                       |  |    |
| <br>                                                            |  |    |
| <220>                                                           |  |    |
| <223> Oligonucleotide primer                                    |  |    |
| <br>                                                            |  |    |
| <400> 23                                                        |  |    |
| ttccggctcg agcgacatcg tgctgtcgca gtctcca                        |  | 37 |
| <br>                                                            |  |    |
| <210> 24                                                        |  |    |
| <211> 32                                                        |  |    |
| <212> DNA                                                       |  |    |
| <213> Artificial Sequence                                       |  |    |
| <br>                                                            |  |    |
| <220>                                                           |  |    |
| <223> Oligonucleotide primer                                    |  |    |
| <br>                                                            |  |    |
| <400> 24                                                        |  |    |
| gagccagagc tcttcagctc cagcttggtc cc                             |  | 32 |
| <br>                                                            |  |    |
| <210> 25                                                        |  |    |
| <211> 35                                                        |  |    |
| <212> DNA                                                       |  |    |
| <213> Artificial Sequence                                       |  |    |
| <br>                                                            |  |    |
| <220>                                                           |  |    |
| <223> Oligonucleotide primer                                    |  |    |
| <br>                                                            |  |    |
| <400> 25                                                        |  |    |
| ttacggccat ggctcagggtt cagctggtcc agtca                         |  | 35 |

<210> 26  
 <211> 35  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Oligonucleotide primer

<400> 26  
 agaccagaga tcttgctcac ggtgaccgtg gtccc 35

<210> 27  
 <211> 79  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Oligonucleotide primer

<400> 27  
 gatctctggc ggccgggtgg cggccgggtgg tgggtcggtgg ggcggccggct cgggtgggtgg 60  
 tgggtcggtggc ggccggccggc 79

<210> 28  
 <211> 79  
 <212> DNA  
 <213> Artificial Sequence

<220>  
 <223> Oligonucleotide primer

<400> 28  
 tcgagccgcc gcccggcggac ccaccaccac ccgagccgcc gccaccggac ccaccaccgc 60  
 ccgagccacc gccaccaga 79

<210> 29  
 <211> 18  
 <212> PRT  
 <213> Artificial Sequence

<220>  
 <223> Linker sequence

<400> 29  
 Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly  
 1 5 10 15  
 Gly Ser

<210> 30  
 <211> 35  
 <212> PRT  
 <213> Artificial Sequence

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<220>
<223> Linker sequence

<400> 30
Gly Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly
1 5 10 15
Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly Gly Ser Gly Gly
20 25 30
Gly Gly Ser
35

<210> 31
<211> 18
<212> PRT
<213> Artificial Sequence

<220>
<223> Linker sequence pKOD2

<400> 31
Gly Leu Glu Gly Ser Pro Glu Ala Gly Leu Ser Pro Asp Ala Gly Ser
1 5 10 15
Asp Ser

<210> 32
<211> 21
<212> DNA
<213> Artificial Sequence

<220>
<223> Oligonucleotide primer

<400> 32
acgacggttg ctgcggcggt c 21

```

```

<210> 33
<211> 21
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> Oligonucleotide primer

```

```

<400> 33
aggctcatta atgatgcggg t 21

```

```

<210> 34
<211> 33
<212> DNA
<213> Artificial Sequence

```

```

<220>
<223> Oligonucleotide primer

```

|                                                                     |     |    |
|---------------------------------------------------------------------|-----|----|
| <400> 34                                                            |     |    |
| ggatccaagg ttacgatcac ggtcatgaac acg                                |     | 33 |
| <210> 35                                                            |     |    |
| <211> 33                                                            |     |    |
| <212> DNA                                                           |     |    |
| <213> Artificial Sequence                                           |     |    |
| <220>                                                               |     |    |
| <223> Oligonucleotide primer                                        |     |    |
| <400> 35                                                            |     |    |
| ctcgagaagg tttaactaaa ttaatacagc gga                                |     | 33 |
| <210> 36                                                            |     |    |
| <211> 783                                                           |     |    |
| <212> DNA                                                           |     |    |
| <213> Streptomyces avidinii                                         |     |    |
| <400> 36                                                            |     |    |
| gagggttcagc tgcagcagtc cggggcagag cttgtggagc caggggcctc agtcaagttg  | 60  |    |
| tcctgcacag cttctggctt caacattaaa gacacctata tgcactgggt gaagcagagg   | 120 |    |
| cctgaacagg gcctggaatg gattggaagg attgatcctg cgaatggtaa tagtaaatat   | 180 |    |
| gtcccgaaatg tccagggcaa ggccactata acagcagaca catcctccaa cacagcctac  | 240 |    |
| ctgcagctca ccagcctgac atctgaggac actgcccgtct attattgtgc tccggttgggt | 300 |    |
| tactacgtgt ctgactatgc tatggcctac tggggtcaag gaacctcagt caccgtctcc   | 360 |    |
| tcaaagatct ctgggtggcg tggctcgggc ggtgggtgggt cgggtggcg cggctcggtt   | 420 |    |
| gttgggtgggt cggggggcg cggctcgagc gacattgtgc tgacccaatc tccagcttct   | 480 |    |
| ttggctgtgt ctcttggca gagggccact atgtcctgca gagccggta aagtgttcat     | 540 |    |
| atttttggcg ttgggtttt gcactggta cagcagaaac caggacagcc acccaaactc     | 600 |    |
| ctcatctatc gtgcataccaa cctagaatct gggatccctg tcaggttcag tggactggg   | 660 |    |
| tctaggacag acttcaccct catcattgtat cctgtggagg ctgtatgtat tgccacctat  | 720 |    |
| tactgtcagc aaactaatga ggatccgtac acgttcggag gggggaccaa gctggaaata   | 780 |    |
| aag                                                                 | 783 |    |
| <210> 37                                                            |     |    |
| <211> 786                                                           |     |    |
| <212> DNA                                                           |     |    |
| <213> Streptomyces avidinii                                         |     |    |
| <400> 37                                                            |     |    |
| gaaggttcagc tgcagcagtc tggggcagaa cttgtgcgtt caggggcctc agtcaaaaatg | 60  |    |
| tcctgcacccg cttctggctt caacattaaa gattactata tgcattgggt gaaacagcgt  | 120 |    |
| ccggaaacagg gcctggaatg gattgggttgg attgatccgg aaaaatggta taccgaatat | 180 |    |
| gcccccggaaat tccagggcaa agccacgtat accaccgata cctcctccaa caccgcctac | 240 |    |
| ctgcagctca gcagcctgac ctctgaagat accgcccgtct attactgtaa taccgtgggt  | 300 |    |
| ctatctacca tgattacgac gcgttgggtt tcgtatgtct ggggcgcagg gaccacggc    | 360 |    |
| accgtctcca agatctctgg tggcggtggc tcggcggtg gtgggtcggtg tggcggtggc   | 420 |    |
| tcgggtgggt gtgggtcggtt cggcggtggc tcgagcgata ttgtgtcgac ccagtctccg  | 480 |    |
| gcttccttaa ccgtatctct gggtctgcgt gccaccatct catgcccgtgc cagaaaaatg  | 540 |    |
| gtcagtgtcat ctggctatag ttatatgtat tggtaaccac agcgtccggg tcagccgccc  | 600 |    |
| aaactccctca tctatcttgc atccaaaccta caatctggta tcccgccccc ttccgtggc  | 660 |    |
| agtgggtctg ggaccgattt caccctcaac atccatccgg tggagaagaaga agatgtcgca | 720 |    |

|                                                                      |     |
|----------------------------------------------------------------------|-----|
| acctattact gtcagcatag tcgtgaactt ccgacgttcg gtgggtggcac caaactggaa   | 780 |
| atcaag                                                               | 786 |
| <br>                                                                 |     |
| <210> 38                                                             |     |
| <211> 771                                                            |     |
| <212> DNA                                                            |     |
| <213> Streptomyces avidinii                                          |     |
| <br>                                                                 |     |
| <400> 38                                                             |     |
| caggtgaagc tgcagcagtc aggtccggag ttgaagaagc cgggtgagac cgtcaagatc    | 60  |
| agctgcaagg cttctggta tacttcacc gtgttggta tgaactgggt gaaggcaggct      | 120 |
| ccgggcagg gtttaaagtg gatgggctgg attaacacca aaactgggtga agcaacctat    | 180 |
| gttgaagagt ttaagggtcg ctttgccttc ttttggaga cctctgcac cactgcctat      | 240 |
| ttgcagatca acaacctcaa aatgaggac acggctaaat atttctgtgc acgttgggac     | 300 |
| ttctatgatt acgtggaagc tatggattac tggggccaag ggaccacggg caccgtctcc    | 360 |
| aagatctctg gtggcggtgg ctggggcggt ggtgggtcg gttggcggtgg ctgggggtgg    | 420 |
| gttgggtcg gggcggtgg ctggggcggt attgtgatga cccagtcac acgtttcatg       | 480 |
| tccacttcag taggtgatcg tgcagcgtc acctgcaaaag ccagtcagaa tgggggtacg    | 540 |
| aatgtgcct ggtatcaaca gaaaccgggt caatccccga aagcactgtat ttactcgca     | 600 |
| ttctaccgtt acagtgggtg cccggatcgc ttcacccggca gtgggtctgg gaccgatttc   | 660 |
| acgctcacca tcagcaatgt acagtctgaa gactggcggt gttttctgg tcatcaat       | 720 |
| tacacctata cggttattcac gttcggtcg gggaccaagt tggaaatgaa g             | 771 |
| <br>                                                                 |     |
| <210> 39                                                             |     |
| <211> 762                                                            |     |
| <212> DNA                                                            |     |
| <213> Streptomyces avidinii                                          |     |
| <br>                                                                 |     |
| <400> 39                                                             |     |
| caggtgaaac tgcagcagtc tgggtcgagaa cttgtgcgtt cagggacaccc agtcaaattt  | 60  |
| tcctgcaccc cttctggctt caacattaa gattcctata tgcattgggt gcgtcagggt     | 120 |
| cggaaacagg gccttggaaat gattgggtgg attgtatccgg agaatgggtga tactgaat   | 180 |
| gcaccgaagt tccaggggcaa agccacctt actaccgata cctccctccaa caccgcctac   | 240 |
| ctgcagctca gcagcctgac ctctgaagat actggcgctt attattgtaa tgaaggact     | 300 |
| ccgactggtc cgtactactt tgattactgg ggtcaaggaa ccacggtcac cgtctccaag    | 360 |
| atctctgggt ggggtggctc gggcggtgg ggtcggtgg gggcggtc ggggtgggt         | 420 |
| gggtcggtgg gggcggtc gagcggaaat gtgcaccc agtctccggc aatcatgtct        | 480 |
| gcacatctccgg gtgagaaatg caccattacc tgcagtgcca gctcaaggtt aagttacatg  | 540 |
| cattgggtcc agcagaaaacc gggtacttcc ccgaaactctt ggatttatacg cacctccaac | 600 |
| ctggcttcgt gtttccggc tgcgttccgtt ggcagtggtt ctggggaccc ttactcttc     | 660 |
| accatcagcc gttatggaaatc tgaagatgtt ggcacttatt actgcccagca acgttagt   | 720 |
| tatccgctca cggttccgtc tggcaccaaa ctggacactga ag                      | 762 |
| <br>                                                                 |     |
| <210> 40                                                             |     |
| <211> 765                                                            |     |
| <212> DNA                                                            |     |
| <213> Streptomyces avidinii                                          |     |
| <br>                                                                 |     |
| <400> 40                                                             |     |
| caggtccaac tacagcagtc agggggagac ttagtgaagc ctggagggtc cctaaaattt    | 60  |
| tcctgtgcag cctctggatt cccttcaat cgctatgcca tgcgttgggt tcgcacact      | 120 |
| ccagagaaga ggctggagtg ggtcgcatcc attagtagtg atggatcgcc ctactatgca    | 180 |
| gacagtgtga agggccgatt caccatctcc agagataatg ccaggaacat cctgtaccta    | 240 |
| caaatacgatca gtctgggttc tgaggacacg ggcacgttatt actgtgcaag agtttattac | 300 |

|            |            |            |            |            |             |     |
|------------|------------|------------|------------|------------|-------------|-----|
| tacggtagta | gttacttga  | ctactgggc  | caaggacca  | cggtcaccgt | gagcaagatc  | 360 |
| totggtggcg | gtggctcgaa | cggtggtggg | tgggtggcg  | gcccgtcgaa | tgggtggcg   | 420 |
| tcggcgccg  | gcggctcgag | cgacatccag | atgactcgt  | ctccaaaatt | catgcccaca  | 480 |
| tcagtaggaa | acagggtcag | cgtcacctgc | aaggccagtc | agaatgcggg | tactaatgt   | 540 |
| gcctggtata | aacagaaacc | agggcaatct | cctaaagcac | tgatttactc | ggcatcgtct  | 600 |
| cggaacagt  | gagtccctga | tcgcttcaca | ggcagtggat | ctgggacaga | tttcaactctc | 660 |
| accatcagca | atgtcagtc  | tgaagactt  | gcagagtatt | tctgtcagca | atataacagc  | 720 |
| tatcctctgg | tcacgttcgg | tgctgggacc | aagctggaaa | taaag      |             | 765 |

&lt;210&gt; 41

&lt;211&gt; 768

&lt;212&gt; DNA

&lt;213&gt; Streptomyces avidinii

|          |            |              |             |             |            |             |     |
|----------|------------|--------------|-------------|-------------|------------|-------------|-----|
| <400> 41 | caggttcagt | tgcagcagtc   | tgatgctgaa  | ttggtgaaac  | cgggtgttcc | agtaaaatt   | 60  |
|          | tcctgcaaag | cttctggcta   | cacccatcacc | gatcatgca   | ttcattgggt | gaaacagaac  | 120 |
|          | ccggAACAGG | gccttggaaatg | gattggttat  | ttctctccgg  | gtaatgtat  | tttcaataac  | 180 |
|          | aatgaacgtt | tcaaaggcaa   | agccacgctg  | accgcagata  | aatcctccag | caccgcctac  | 240 |
|          | gtgcagctca | acagcctgac   | gtctgaagat  | tctgcagtgt  | atttctgtac | gcgttccctg  | 300 |
|          | aatatggcct | actggggtca   | aggtacctca  | gtcaccgtct  | ccaagatctc | tgggtggcggt | 360 |
|          | ggctcgccg  | gtgggtggtc   | gggtggcgcc  | ggctcggtgt  | gtgggtggtc | ggggggcgcc  | 420 |
|          | ggctcgagcg | atattgtat    | gtcacagtc   | ccgttctccc  | taccgggtgc | agttggcgaa  | 480 |
|          | aaagttacct | ttagctgca    | atccagtcag  | agccctttat  | atagtggtaa | tcaaaaaaac  | 540 |
|          | tacttggcct | ggtaccagca   | gaaaccgggt  | cagtcctccg  | aactgtctat | ttacttggca  | 600 |
|          | tccgctcg   | aatctgggtt   | cccgatcgc   | ttcacccggca | gtggttctgg | gaccgatttc  | 660 |
|          | accctctcca | tcagcagtgt   | gaaaaccgaa  | gacctggcag  | tttattactg | tcagcagtat  | 720 |
|          | tatagctatc | cgctcacgtt   | cggtgtcgaa  | acccaaactgg | tgctgaag   |             | 768 |

&lt;210&gt; 42

&lt;211&gt; 765

&lt;212&gt; DNA

&lt;213&gt; Streptomyces avidinii

|          |             |              |            |             |             |             |     |
|----------|-------------|--------------|------------|-------------|-------------|-------------|-----|
| <400> 42 | gaagtggaaac | ttgaagagtc   | tgggtgtggc | ttggtgcaac  | cgggtggctc  | catgaaaactc | 60  |
|          | tcttggctg   | cttctggctt   | cacctttatg | gatccctgaa  | tggattgggt  | ccggccagtc  | 120 |
|          | ccggagaaag  | ggcttggaaatg | ggttgcgtaa | attcgtaaca  | aagccaaataa | tcatggtacc  | 180 |
|          | tattatgtat  | agtctgtgaa   | agggcgctc  | accatctcac  | gtgtatgattc | caaaagtcgt  | 240 |
|          | gtgtacctgc  | aaatgattag   | cttacgtgt  | gaagataccg  | ggctttatata | ctgtaccggg  | 300 |
|          | gaatttgctt  | actggggcca   | ggggacgctg | gtcaccgtct  | ctaagatctc  | tgggtggcggt | 360 |
|          | ggctcgccg   | gtgggtggtc   | gggtggcgcc | ggctcggtgt  | gtgggtggtc  | ggggggcgcc  | 420 |
|          | ggctcgagcg  | atgttgcgtat  | gacccaaact | ccgttctccc  | tgccggtcac  | tcttggtgat  | 480 |
|          | caagcttcca  | tctcttgcgg   | ttcttagtgc | aacccatgtac | ataacaatgg  | taacacat    | 540 |
|          | ttatattgtt  | tcctgcagaa   | atcaggccag | tctccgaaac  | tgctgatata  | tcgcgcaccc  | 600 |
|          | atccgcttt   | ctgggttccc   | ggatcgctc  | agtggcagtg  | gttcagaaac  | cgatttcacg  | 660 |
|          | ctcaagatca  | gccgtgtgga   | agctgaagac | ctgggtgttt  | atttctgttt  | tcaaggtacg  | 720 |
|          | catgttccgt  | ggacgttcgg   | tggtggcacc | aaactggaaa  | tcaag       |             | 765 |

&lt;210&gt; 43

&lt;211&gt; 741

&lt;212&gt; DNA

&lt;213&gt; Streptomyces avidinii

<400> 43

|             |             |            |            |            |            |     |
|-------------|-------------|------------|------------|------------|------------|-----|
| caggtgcagc  | ttcaggagtc  | aggacctggc | cttgtgaaac | cctcacagtc | actctccctc | 60  |
| acctgttccg  | tcactggta   | ctccatca   | actgattact | ggggctggat | ccggaagttc | 120 |
| ccagggaaata | aatggagtg   | gatgggatac | ataagctaca | gtggtagcac | tggctacaac | 180 |
| coatctctca  | aaagtcgaat  | ctccattact | agagacacat | cgaagagtca | gttcttcctg | 240 |
| cagttgaact  | ctgttaactac | tgaggacaca | gccacatatt | actgtgcaag | atacagtagc | 300 |
| cttgattact  | ggggccgagg  | agtcatggtc | gcagtcctca | agatctctgg | tggcggtggc | 360 |
| tcggcgggtg  | gtgggtcggg  | tggcggcggc | tcgggtggt  | gtgggtcggg | cggcggcggc | 420 |
| tcgagcgatg  | ttgtgatgac  | ccagacacca | ccgtctttgt | cggttgcac  | tggacaatca | 480 |
| gtctccatct  | cttgcaagtc  | aagttagagc | ctcgatata  | gtgatggaaa | gacatatttg | 540 |
| cattggttat  | tacagagtcc  | tggcaggct  | ccgaagcgcc | taatctatca | ggtgtctaat | 600 |
| ctgggctctg  | gagtccctga  | caggttcagt | ggcactggat | cacagaaaga | ttttacactt | 660 |
| aaaatcagoa  | gagtggaggg  | tgaggattt  | ggagttact  | actgcgcgca | aactacacat | 720 |
| tttcctctca  | cgttcggttc  | g          |            |            |            | 741 |

<210> 44

<211> 765

<212> DNA

<213> *Streptomyces avidinii*

<400> 44

|            |             |            |            |            |            |     |
|------------|-------------|------------|------------|------------|------------|-----|
| cagtttcagc | tggtccagtc  | aggggctgag | ctggtaagc  | ctggggcctc | agtgaagatg | 60  |
| tcctgcaagg | tttctggcta  | cacatttacc | agttacaata | tgcactgggt | aaagcagaca | 120 |
| cctggacagg | gcctggaatg  | gattggagct | atttatccag | gaaatggta  | tacttcctac | 180 |
| aatcagaagt | tcaaaggcaa  | ggccacattg | actgcagaca | aatcctccag | cacagcctac | 240 |
| atgcagctca | gcagcctgac  | atctgaggac | tctcggtct  | attactgtgc | aagagcgcaa | 300 |
| ttacgaccta | actactggta  | ttcgatgtc  | tggggcgcag | ggaccacggt | caccgtgagc | 360 |
| aagatctctg | gtggcgggtgg | ctcggcgggt | ggtgggtcgg | gtggcggcgg | ctcgggtgg  | 420 |
| ggtgggtcgg | gcggcggcgg  | ctcgagcgac | atcggtctgt | cgcagtctcc | agcaatcctg | 480 |
| tctgcatctc | caggggagaa  | ggtcacaatg | acttgcaggg | ccagctcaag | tgtaagttac | 540 |
| atgcactgg  | accagcagaa  | gccaggatcc | tcccccaaac | cctggattta | tgccacatcc | 600 |
| aacctggctt | ctggagtccc  | tgctcgctc  | agtggcagtg | ggtctggac  | ctcttactct | 660 |
| ctcacaatca | gcagagtgg   | ggctgaagat | gctgccactt | attactgcca | gcagtggatt | 720 |
| agtaacccac | ccacgttcgg  | tgctgggacc | aagctggagc | tgaag      |            | 765 |

<210> 45

<211> 765

<212> DNA

<213> *Streptomyces avidinii*

<400> 45

|             |             |            |            |            |            |     |
|-------------|-------------|------------|------------|------------|------------|-----|
| cagtttcagc  | tgcaacagcc  | aggggctgag | ctggtaagc  | ctggggcctc | agtgaagatg | 60  |
| tcctgcaagg  | tttctggcta  | cacatttacc | agttacaata | tgcactgggt | aaagcagaca | 120 |
| cctggacagg  | gcctggaatg  | gattggagct | atttatccag | gaaatggta  | tacttcctac | 180 |
| aatcagaagt  | tcaaaggcaa  | ggccacattg | actgcagaca | aatcctccag | cacagcctac | 240 |
| atgcagctca  | gcagcctgac  | atctgaggac | tctcggtct  | attactgtgc | aagaagcacc | 300 |
| tattacggcg  | gtgattggta  | ttcaacgtc  | tggggcgcag | ggaccacggt | caccgtgagc | 360 |
| aagatctctg  | gtggcgggtgg | ctcggcgggt | ggtgggtcgg | gtggcggcgg | ctcgggtgg  | 420 |
| ggtgggtcgg  | gcggcggcgg  | ctcgagccag | atcggtctgt | cgcagtctcc | agcaatcctg | 480 |
| tctgcatctc  | caggggagaa  | ggtcacaatg | acttgcaggg | ccagctcaag | tgtaagttac | 540 |
| attcaactgtt | ttcagcagaa  | gccaggatcc | tcccccaaac | cctggattta | tgccacatcc | 600 |
| aacctggctt  | ctggagtccc  | tgtgcgcctc | agtggcagtg | ggtctggac  | ctcttactct | 660 |
| ctcacaatca  | gcagagtgg   | ggctgaagat | gctgccactt | attactgcca | gcagtggacc | 720 |
| agtaacccac  | ccacgttcgg  | tgctgggacc | aagctggaga | tcaag      |            | 765 |

<210> 46  
 <211> 780  
 <212> DNA  
 <213> Streptomyces avidinii

<400> 46

|            |             |             |             |             |             |     |
|------------|-------------|-------------|-------------|-------------|-------------|-----|
| caggttcagc | tggtggaaatc | aggagggtggc | ctgggtgcagc | ctggaggatc  | cctgaaaactc | 60  |
| tcctgtgcag | cctcaggatt  | cgatttcagt  | agataactgg  | tgagttgggt  | ccggcaggct  | 120 |
| ccagggaaag | ggctagaatg  | gattggagag  | attaatccaa  | ctagcagtag  | gataaaacttt | 180 |
| acgccatctc | taaaggataa  | agtcttcatc  | tccagagaca  | acgccaaaaaa | tacgctgtac  | 240 |
| ctgcaaatga | gcaaagttag  | atccgaggac  | acagccctt   | attactgtgc  | aagagggAAC  | 300 |
| tactataggt | acggagatgc  | tatggactac  | tgggtcaag   | gaacctcagt  | caccgtgagc  | 360 |
| aagatctctg | gtggcggtgg  | ctcgccgggt  | ggtgggtcgg  | gtggcggtgg  | ctcgccgggt  | 420 |
| ggtgggtcgg | gcggcgccgg  | ctcgagcgac  | atcgtgctga  | cccagtctcc  | tgcttcctta  | 480 |
| gctgtatctc | tgggacagag  | ggccaccatc  | tcatgcaggg  | ccagcaaaag  | tgtcagtaca  | 540 |
| tctggctata | gttatctgca  | ctggtaccaa  | cagaaaccag  | gacagccacc  | caaactcttc  | 600 |
| atctatcttg | catccaacct  | agaatctgg   | gtccctgcca  | ggttcagtg   | cagtgggtct  | 660 |
| gggacagact | tcaccctcaa  | catccatctc  | gtggaggagg  | aggatgtgc   | aacctattac  | 720 |
| tgtcagcaca | gtagggagct  | tccattcacg  | ttcggctcgg  | ggacaaagtt  | ggaaataaaag | 780 |